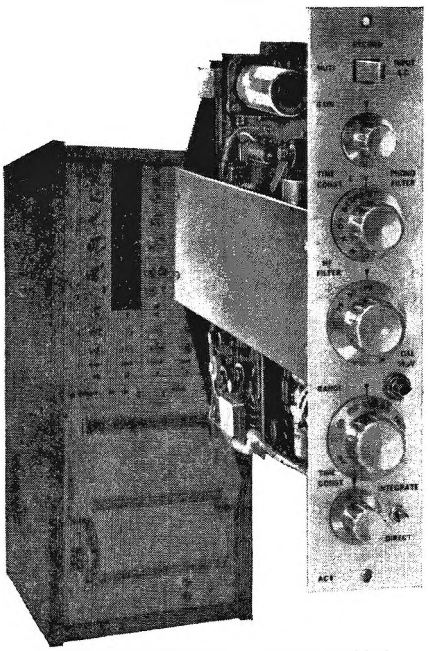
# Journal of Pharmacy and Pharmacology


The Pharmaceutical Society of Great Britain

Volume 21 Number 7 July 1969

# A NEW EMG INTEGRATOR



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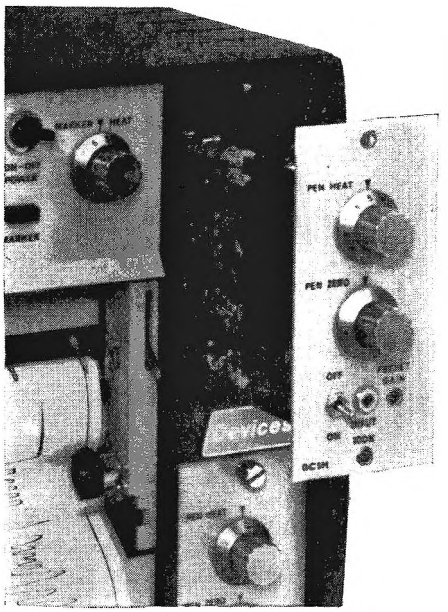


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# Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN
17 Bloomsbury Square, London, W.C.1. Telephone: 01-405 8967
**Volume 21 Number 7** **July 1969**



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*J. Pharm. Pharmac.*, 1969, 21, 409–414 *Received March* 20, 1969

# An automated apparatus for dissolution studies

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An automated apparatus for dissolution rate studies of solid dosage forms under sink conditions at constant volume has been developed. Sink condition was maintained in the small volume of the buffer by continuous elimination of the solution and replacement with fresh buffer. A mathematical treatment of the dissolution-dilution system for the apparatus is given. The validity of the calculations has been proved using clomipramine solution and tablets.

The *in vitro* release of drug from solid dosage forms has been subject to intensive studies in recent years (Morrison & Campbell, 1965). A variety of techniques is used to determine *in vitro* dissolution rates of medicaments from these dosage forms. Hersey (1968) has classified the methods in three groups: (*a*) natural convection, non-sink methods, (*b*) forced convection, non-sink methods, (*c*) forced convection, sink methods.

Noyes & Whitney (1897) had derived a differential equation for a dissolving substance in a medium as

$$\frac{dc}{dt} = KS(C_s - C), \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

where dc/dt is the rate at which the substance is dissolving across a surface area S, and $(C_s - C)$ is the concentration gradient between $C_s$, the concentration of substance in a thin saturated liquid film adjacent to the dissolving surface and C the concentration in the surrounding bulk medium. K is a rate constant for dissolution incorporating the diffusion coefficient and the film thickness. If in the above equation the concentration gradient $C_s - C$ tends towards $C_s$, the retarding effect on the solubility of the substance becomes negligible. Equation (1) may be rewritten as

$$\frac{dc}{dt} = KSC_s \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

This is known more generally as sink condition and may be achieved by removal of the solute from the dissolving medium. The methods to obtain sink conditions are: use of (*a*) an adsorbent, (*b*) an organic phase, (*c*) dialysis.

We have sought to develop an automated apparatus for dissolution rate studies under sink conditions and to find a way for maintaining sink conditions in a single phase system of small volume.

## EXPERIMENTAL

### *Description of the apparatus*

Two thermostated glass cylinders $C_1$ and $C_2$ (diameter 45 mm) are connected to each other with a tube A in such a way that the dissolution medium can flow from

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$C_1$ to $C_2$. A constant volume of dissolution medium is maintained by means of an overflow system S. This consists of an adjustable tube $S_1$ connected to a pump $P_1$ leading to the reservoir $S_2$. The replacement of dissolution medium in $S_2$ is by the pump $P_1$ from the main reservoirs $S_3$ and $S_4$ respectively.

Tube A is connected to a vacuum pump by means of a T-shaped tap, which permits a rapid removal of dissolution medium from either cylinder or from both. Cylinder $C_2$ is equipped with a four blade stirrer R (diameter 15 mm) with adjustable speed to maintain a homogeneous solution. From this cylinder the solution is pumped off continuously by pump $P_2$ (Beckman solution metric pump) through tube B, filtered at F (Millipore type SC) to remove foreign particles, and the absorbance is continuously measured and recorded in the spectrophotometer Sp (Beckman DB-G; W+W recorder type 202). Fig. 1 illustrates the set-up.

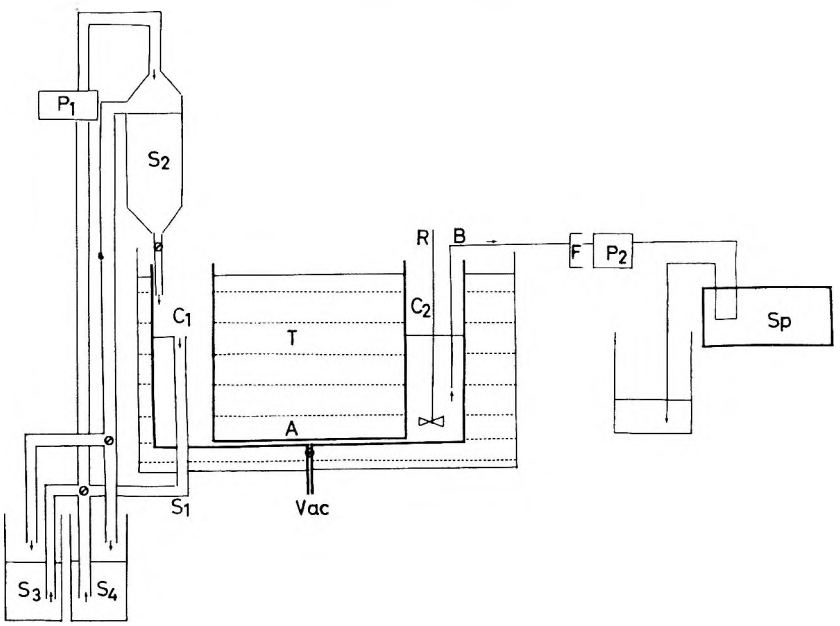


FIG. 1. Design of the apparatus for dissolution studies. $C_1$, reservoir cylinder. $C_2$, testing cylinder. $P_1$, $P_2$, pumps. $S_1$–$S_4$, overflow system. A, B, tubes. R, stirrer. F, filter. Sp, spectrophotometer. Vac., vacuum. T, const. temp. bath.

For the simultaneous determination of several dissolution rates a number of testing cylinders $C_2$ can be connected to cylinder $C_1$. All studies were made at 37 ± 0·1°.

*Procedure*

*Elimination of dissolved drug.* A concentrated solution containing a known amount of drug is injected in cylinder $C_2$ containing 150 ml of distilled water. The solution is stirred at a selected rate and the pump is adjusted to remove the solution at a fixed flow rate.

Clomipramine [3-chloro-5-(3-dimethylaminopropyl)-10,11-dihydro-5*H*-dibenz[b,f]-azepine hydrochloride; Anafranil, GEIGY] was the drug used in the present work. The stirring rate was 200 rev/min and the flow rates were: 2, 5, 8, 11 and 14 ml/min.

*Dissolution of drug from tablets.* Artificial gastric juice (USP, without enzymes) is adjusted to a volume of 150 ml in cylinder $C_2$. A tablet to be tested is added.

The flow rate of the eliminating pump is adjusted to a suitable rate and the speed of the stirrer maintained at a constant rate. The absorbance is continuously recorded at a chosen wavelength.

For the present study the flow rate was 2 ml/min, the stirring rates 50, 100, 200, 400 rev/min and the wavelength 252 nm.

*Calculation*

*Rate of elimination of dissolved drug under constant volume condition.* Consider that $c_o$ is the amount of dissolved drug at time Zero and E the rate of elimination of the solution at constant volume. Hence

$$E = \frac{F}{V} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

where $F$ = flow rate in ml/min
$V$ = constant volume in cylinder $C_2$ in ml

The rate of change of concentration in the solution can be expressed as

$$-\frac{dc}{dt} = E \cdot c \quad .. \quad .. \quad .. \quad .. \quad (4)$$

By integrating between the limits $c_o$ and $c_t$ the above equation becomes

$$\int_{c_o}^{c_t} \frac{dc}{c} = -E \int_{o}^{t} dt \quad .. \quad .. \quad .. \quad .. \quad .. \quad (5)$$

and therefore

$$c_t = c_o e^{-Et} \quad .. \quad .. \quad .. \quad .. \quad (6)$$

The rate of change of concentration in the solution under constant volume condition is a first order kinetic.

*Calculation of drug dissolved from solid dosage forms.* This calculation has been done assuming the time interval $\Delta t$ is small and at no time exceeds 2 min.

The amount of drug released after $\Delta t$ in a given volume will be

$$C_1 = c_1 + c_1 \Delta t E \quad .. \quad .. \quad .. \quad .. \quad (7)$$

where $c_1$ = amount of drug observed in the solution;

$c_1 \Delta t E$ = amount of drug eliminated from the solution during the interval $\Delta t$.

After the next interval $\Delta t$ the amount released is calculated similarly,

$$C_2 = c_2 + c_1 \Delta t E + c_2 \Delta t E$$
$$C_2 = c_2 + \Delta t E (c_1 + c_2) \quad .. \quad .. \quad .. \quad (8)$$

and so on, until the peak of the curve is reached, where the amount of drug released will be

$$C_p = c_p + \Delta t E (c_1 + c_2 \ldots c_p)$$

$$C_p = c_p + \Delta t E \sum_{i=1}^{i=p} c_1 \quad .. \quad .. \quad .. \quad .. \quad (9)$$

Thus the amount of drug in the solution is equal to $c_p$.

When there will be no further release of drug from the dosage form, the elimination of the dissolved drug will follow equation (6). However, if the amount observed after the next interval of time is higher than the one calculated according to equation (6), the difference will show a further amount of drug dissolved during this interval.

Thus the drug released after the peak may be calculated for $\Delta t$ as

$$C_{p+1} = c_{p+1} - c_p\, e^{-E\Delta t} \quad .. \quad .. \quad .. \quad .. \quad (10)$$

where $C_{p+1}$ = amount of drug released in first interval after peak, $c_{p+1}$ = amount observed in the solution, $c_p\, e^{-E\Delta t}$ = amount of drug expected by pure dilution according to equation (6) for interval $\Delta t$. The release during the next interval will be

$$C_{p+2} = c_{p+2} - c_{p+1}\, e^{-E\Delta t}$$

or in general form

$$C_{p+n} = c_{p+n} - c_{p+(n-1)}\, e^{-E\Delta t} \quad .. \quad .. \quad .. \quad (11)$$

When

$$c_{p+n} = c_{p+(n-1)}\, e^{-E\Delta t} \quad .. \quad .. \quad .. \quad (12)$$

equation (11) becomes

$$C_{p+n} = 0 \quad .. \quad .. \quad .. \quad .. \quad (13)$$

Thus, at the interval (p + n) the amount observed in the solution being equal to the amount expected by pure dilution, there is no further release of drug.

Hence the total amount of drug released will be

$$C_T = C_p + \sum_{q=p+1}^{q=p+n} C_q \quad .. \quad .. \quad .. \quad .. \quad (14)$$

$C_T$ = Total release, $C_p$ = release of drug before peak, $C_q$ = release of drug after peak. These calculations are conveniently done with an electronic desk-top computer Olivetti Programma 101 according to equations (9) and (14).

## RESULTS

### *Elimination of dissolved drug*

The results obtained with the different flow rates plotted on a semilog graph of rest concentration against time showed straight lines with the slopes $-E/2{\cdot}303$.

The experimental and calculated results are compared in Table 1.

### *Dissolution of drug from clomipramine tablets*

The dissolution of drug from tablets containing 25 mg at stirring rates 50, 100, 200, 400 rev/min was examined. At the stirring rate of 50 rev/min a significantly different dissolution behaviour was observed compared with the higher stirring rates. As the results at the rates 200 and 400 rev/min were close, 200 rev/min was used. The results are in Fig. 2.

Tablets containing 11 mg of drug were tested. The results were obtained for a single tablet as well as for three tablets per run. The ultraviolet absorbance for one tablet being approx. 0·3, three tablets were chosen for the determination of average release and control of sink condition. The same effect may be achieved by decreasing the buffer volume. The results are in Fig. 3. Spectroscopic analysis was made in 0·1N HCl.

Table 1. *Comparison of experimental and calculated rest concentrations of dissolved drug under constant volume condition.* Dose 20 mg, volume 150 ml of artificial gastric juice without enzymes USP

| Flow rate ml/min | Time (min) | Drug in solution in mg | | Slope of the plot |
|---|---|---|---|---|
| | | experimental* | calculated | |
| 2 | 40 | 11·7 | 11·9 | 0·0058 |
| | 80 | 7·0 | 7·1 | |
| | 120 | 4·2 | 4·2 | |
| | 160 | 2·4 | 2·5 | |
| | 200 | 1·4 | 1·5 | |
| | 240 | 0·7 | 0·9 | |
| | 280 | 0·4 | 0·6 | |
| | 300 | 0·2 | 0·4 | |
| 5 | 20 | 10·1 | 10·2 | 0·0147 |
| | 40 | 5·2 | 5·2 | |
| | 60 | 2·7 | 2·7 | |
| | 80 | 1·3 | 1·4 | |
| | 100 | 0·5 | 0·7 | |
| | 120 | 0·2 | 0·4 | |
| 8 | 20 | 6·8† | 6·7 | 0·0238 |
| | 40 | 2·4† | 2·3 | |
| | 60 | 0·8 | 0·8 | |
| | 70 | 0·4 | 0·5 | |
| 11 | 10 | 9·8† | 9·4 | 0·0331 |
| | 20 | 4·5† | 4·4 | |
| | 30 | 2·1 | 2·1 | |
| | 40 | 0·9 | 1·0 | |
| | 50 | 0·3 | 0·5 | |
| 14 | 10 | 7·6† | 7·5 | 0·0426 |
| | 20 | 2·8 | 2·8 | |
| | 30 | 0·9 | 1·1 | |
| | 40 | 0·3 | 0·5 | |

* Mean of three determinations.

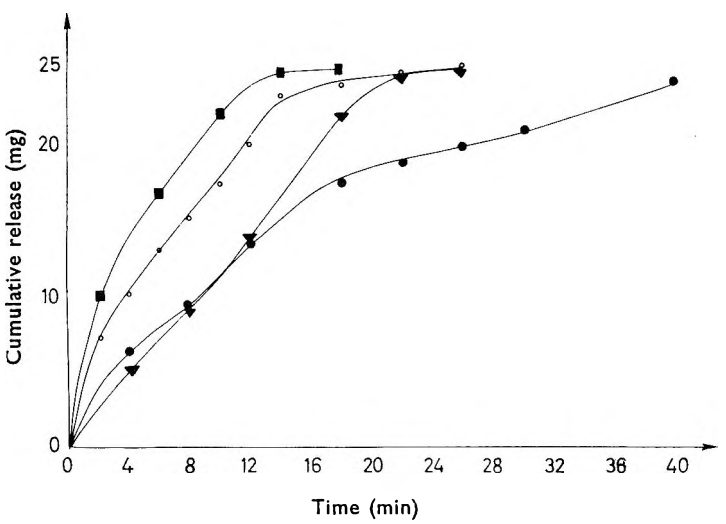


FIG. 2. Cumulative release of clomipramine from 25 mg tablets at different stirring rates. ● 50, ▼ 100, ○ 200, □ 400 rev/min.

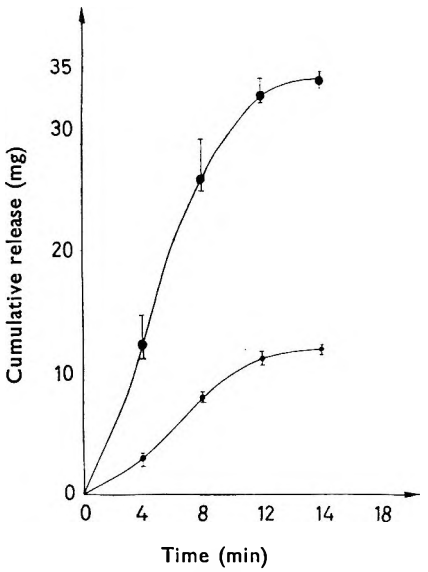


FIG. 3. Cumulative release of clomipramine from 11 mg tablets. ● 3 tablets (mean). • 1 tablet (mean). I maximum–minimum of single values.

From the above results it can be concluded that the apparatus provides reproducible results under sink condition.

## DISCUSSION

In the present method it is easy to change from one buffer solution to another either by continuous change or by immediate vacuum removing and refilling. In the method discussed by Pernarowski, Woo & Searl (1968) only the continuous change is possible. The sink condition depends on the flow rate of the pump which may be adjusted if required. However, if at any moment the concentration of drug in solution goes beyond sink condition, the rapid removal and refilling of buffer may be helpful. The method of calculation remains the same.

## REFERENCES

HERSEY, J. A. (1968). Brit. Pharm. Conference, Discussion Forum.
MORRISON, A. B. & CAMPBELL, J. A. (1965). *J. pharm. Sci.*, **54**, 1–8.
NOYES, A. A. & WHITNEY, W. R. (1897). *J. Am. chem. Soc.*, **19**, 930.
PERNAROWSKI, M., WOO, W. & SEARL, R. O. (1968). *J. pharm. Sci.*, **57**, 1419–1421.

*J. Pharm. Pharmac.*, 1969, **21**, 415–422 *Received January* 13, 1969

# The relation between the bactericidal activities and certain physico-chemical properties of some fluorophenols

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The antibacterial activities, as demonstrated by the Rideal-Walker coefficients and viable counts, of mono-, 2,4,5- tri-, 2,3,5,6- tetra- and pentafluorophenol increase with the number of substituent fluorine atoms. The thermodynamic activities (Ferguson values) range from 0·083 to 0·022, and indicate a non-specific physical mode of action. Linear correlations occur between the equitoxic concentrations of the compounds to *Escherichia coli* (99·9% mortality after 50 min contact) and their molecular weights, number of fluorine substituents and water solubilities. A similar relation exists between the molar concentrations at the Rideal-Walker end points and the oleyl alcohol-water partition coefficients; no correlation occurs between toxicity and the cyclohexane-water partition coefficients. The surface tensions of equitoxic solutions vary between 57·5 and 63·2 $mNm^{-1}$ (dynes/cm).

The bactericidal activities of *o*-chloro-, 2,4-dichloro- and 2,4,6-trichlorophenol, increase with increasing halogen substitution (Klarmann, Shternov & von Wowern, 1929; Ordal, 1941; Sykes, 1965). The low solubilities of tetra- and pentachlorophenol may interfere with the demonstration of their intrinsic activities (Wolf & Westveer, 1952), but using an aqueous suspension of organisms, Allawala & Riegelman (1954) demonstrated increasing toxicity throughout the chlorophenol series. The antibacterial activities of the monofluorophenols have been reported by Pinney & Walters (1967). This paper describes the activities of some polyfluorinated phenols and the relations between their toxicities and certain physico-chemical properties.

## EXPERIMENTAL

### *Materials*

Phenol A.R. (B.D.H.) and *o*-, *m*- and *p*-fluorophenols (Koch Light Limited) as described by Pinney & Walters (1967). 2,4,5-trifluorophenol, 2,3,5,6-tetrafluorophenol and pentafluorophenol (Imperial Smelting Corporation Ltd.). Their purity was confirmed by gas chromatography using the system of Pinney & Walters (1967) with nitrogen as the carrier gas.

### *Methods*

*Organisms and bactericidal evaluations.* *Salmonella typhi* (NCTC 786) for Rideal-Walker evaluations and *Escherichia coli* (NCTC 5933), in aqueous suspension, for viable counts as described by Pinney & Walters (1967).

*Solubility.* Excess of each phenol was added to separate 20 ml quantities of distilled water in 50 ml "Quickfit" conical flasks. The flasks were held at 40° for

* This work formed part of a thesis submitted for the degree of Doctor of Philosophy in the University of London.

1 h and then shaken 100 times/min at 25° for 36 h. After standing at 25°, the supernatant aqueous phases were removed and further clarified by centrifugation. The solutions were suitably diluted for spectrophotometric assay. Calibration curves obeyed Beer's Law and the respective $\lambda_{max}$ of the phenols in water are given in Table 2.

*Phase distribution.* (*a*) Cyclohexane–water. Amounts of 20 ml of approximately $10^{-3}$M solutions of each phenol and 20 ml of cyclohexane in 50 ml "Quickfit" flasks were shaken 20 times/min at 25° for 24 h. The aqueous phases were separated after standing at 25° for 1 h and the concentrations of phenol in solution determined spectrophotometrically. Water similarly treated with cyclohexane, was used as the reference solvent. The apparent partition coefficients (Reese, Irwin & others, 1964) were calculated from the respective concentrations in the two phases. The results are the means of duplicate experiments.

(*b*) Oleyl alcohol–water. Amounts of $5 \times 10^{-3}$M solutions ($5 \times 10^{-2}$M for pentafluorophenol) and oleyl alcohol were used as above.

*Surface tension.* Deionized water containing 0·1% potassium permanganate and 0·1% sodium hydroxide was double distilled in an all glass apparatus. Distillate having a surface tension greater than 71 $mNm^{-1}$ (dynes/cm) was collected. Solutions of the phenols were prepared with this water using glass apparatus which had been degreased with 1·0% sodium nitrate in sulphuric acid, rinsed with double distilled water and dried at 105°.

Surface tensions were determined by the Wilhelmy plate method using an apparatus devised by Padday (1957) and modified by Warburton (1966).

## RESULTS

The log survivor-time curves for *E. coli* in aqueous solutions of the polyfluorophenols are shown in Fig. 1. The times to reduce the viability by 99·9% were obtained from these curves, and plots of the logarithms of these times against the log molar concentration of bactericide were linear (Fig. 2). The slopes of the lines in Fig. 2 gave the concentration exponents for the respective compounds (Table 1). The concentrations of phenol and the fluorophenols which reduce the initial viable population by 99·9% in 50 min were calculated from the data in Fig. 2. Table 1 gives the ratios of phenol : fluorophenol for these concentrations. The Rideal-Walker coefficients of the compounds were also determined and are presented in Table 1.

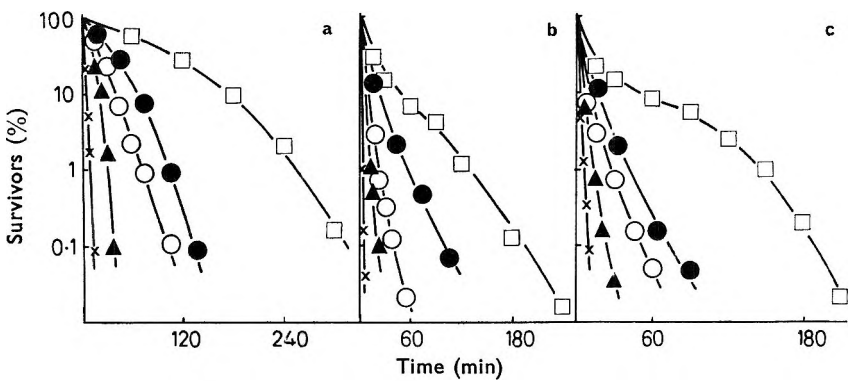


FIG. 1. Log survivor-time curves for *E. coli* exposed to fluorophenols.
a. Trifluorophenol (%): × = 0·4. ▲ = 0·3. ○ = 0·275. ● = 0·25. □ = 0·225. b. Tetrafluorophenol (%): × = 0·25. ▲ = 0·2. ○ = 0·175. ● = 0·15. □ = 0·125. c. Pentafluorophenol (%): × = 0·2. ▲ = 0·15. ○ = 0·125. ● = 0·1125. □ = 0·1.

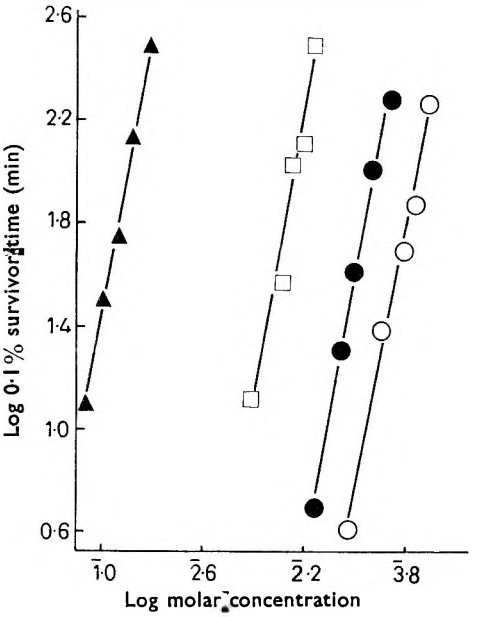


FIG. 2. Concentration exponent plots for phenol and fluorophenols against *E. coli*. ▲ = phenol. □ = trifluorophenol. ● = tetrafluorophenol. ○ = pentafluorophenol.

Table 1. *Comparison of the relative bactericidal activities of phenol and fluorophenols by viable counting and Rideal-Walker techniques*

| Compound | Concentration exponent | Concn for 99·9% mortality of *E. coli* in 50 min at 25° % | Ratio: concn phenol / concn fluorophenol | Rideal-Walker Coefficient |
|---|---|---|---|---|
| Phenol | 6·2 | 0·83 | 1·0 | 1·0 |
| Trifluorophenol | 5·5 | 0·30 | 2·8 | 2·5 |
| Tetrafluorophenol | 5·4 | 0·16 | 5·2 | 3·4 |
| Pentafluorophenol | 5·1 | 0·12 | 6·9 | 4·3 |

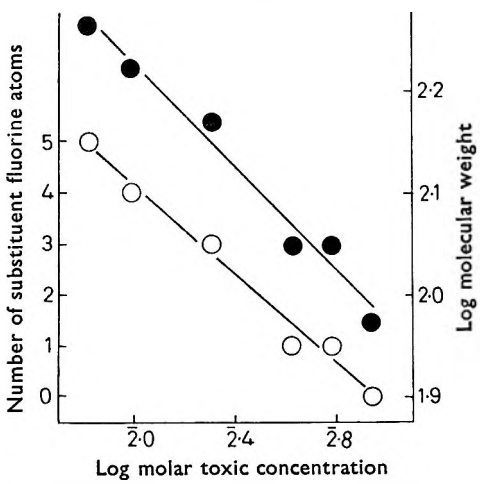


FIG. 3. Correlation of log molar concentrations of fluorophenols which produce 99·9% mortality in 50 min with their log molecular weights (●) and with the number of fluorine atoms substituted in the phenol molecule (○).

The correlation coefficients relating the log molar toxic concentration (99·9% mortality in 50 min) with the number of substituent fluorine atoms and with the log molecular weights (Fig. 3) are 0·991 and 0·987 respectively (d.f. = 6) The regressions are linear since the calculated coefficients correspond to $P'$ values $< 0{\cdot}001$.

Table 2. *Solubilities and thermodynamic activities of toxic solutions of phenol and fluorophenols.* (*Equitoxic concentration is that which produces* 99·9% *mortality of* E. coli *in* 50 *min at* 25°)

| Compound | $\lambda_{max}$ mμ | Solubility mol/litre at 25° (So) | Equitoxic concn mol/litre (St) | Thermodynamic activity of toxic solution St/So |
|---|---|---|---|---|
| Phenol | 270 | 0·93 | 0·088 | 0·095 |
| *o*-Fluorophenol | 267 | 0·72 | 0·060 | 0·083 |
| *m*-Fluorophenol | 267·5 | 0·69 | 0·042 | 0·061 |
| *p*-Fluorophenol | 277 | 0·72 | 0·060 | 0·083 |
| Trifluorophenol | 274 | 0·42 | 0·020 | 0·048 |
| Tetrafluorophenol | 226·5 | 0·37 | 0·0096 | 0·026 |
| Pentafluorophenol | 265 | 0·30 | 0·0066 | 0·022 |

The log molar solubility (from Table 2) plotted against log molar toxic concentration is linear (Fig. 4) with a correlation coefficient of 0·985 (d.f. = 6; $P' < 0{\cdot}001$). Ferguson (1939) cites such linear correlations as being indicative of a physical mode of action. The relatively high thermodynamic activities of the toxic solutions (Table 2), calculated according to the method of Ferguson (1939), confirm physical toxicity.

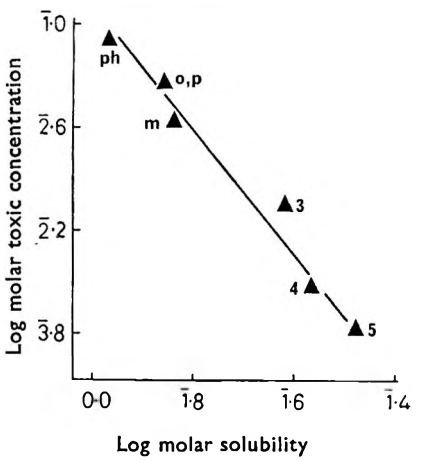


FIG. 4. Relation between log molar solubility of fluorophenols and their log molar toxic concentration (99·9% mortality in 50 min). Phenol = ph; monofluorophenols: *ortho* = o, *meta* = m, *para* = p; trifluorophenol = 3, tetrafluorophenol = 4, pentafluorophenol = 5.

The apparent partition coefficients of the compounds between cyclohexane (a solvent which does not form hydrogen bonds with the solute) and water (Table 3) are low; there is no correlation between them and the bactericidal activities which increase with increasing fluorine substitution (Fig. 3). However, with oleyl alcohol (a proton-attracting solvent), the antibacterial activities increase with the partition coefficients. The plot of toxic concentration against partition coefficient for oleyl alcohol (Fig. 5) is linear with a correlation coefficient of 0·990 (d.f. = 6; $P' < 0{\cdot}001$).

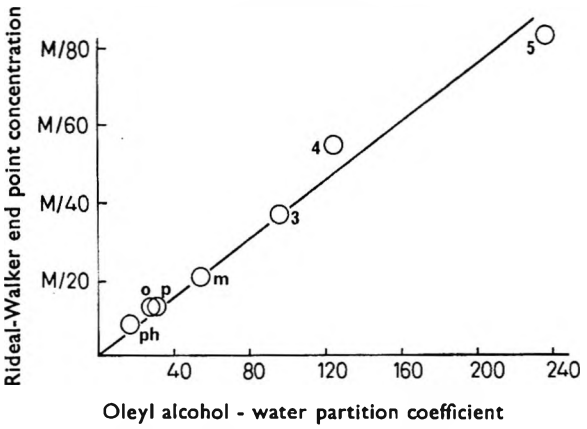


FIG. 5. Relation between toxic concentration of fluorophenols and their partition coefficients in oleyl alcohol-water. Symbols as in Fig. 4.

The plots of surface tension against concentration (not shown) for each phenol were concave to the axes. The surface tensions (Table 3) of equitoxic aqueous solutions of the bactericides were read from these plots. It is apparent that the concentration of each compound necessary to produce a 99·9% kill of *E. coli* in 50 min reduces the surface tension of water to the same order of magnitude. The mean surface tension of such equitoxic solutions is 60·3 $mNm^{-1}$, with limits of error ($P' = 0{\cdot}05$) of $\pm$ 2·0, and a coefficient of variation of 3·6%.

Table 3. *Partition coefficients and surface activities of phenol and fluorophenols.* (*Concentration of toxic solutions as in Table* 2)

| Compound | Apparent partition coefficients at 25° | | Surface tension of equitoxic solution at 25° |
|---|---|---|---|
| | Cyclohexane–water | Oleyl alcohol–water | $mNm^{-1}$ (dynes/cm) |
| Phenol | 0·1 | 15·6 | 60·4 |
| *o*-Fluorophenol | 0·7 | 24·8 | 58·8 |
| *m*-Fluorophenol | 0·2 | 54·1 | 63·0 |
| *p*-Fluorophenol | 0·1 | 29·9 | 58·6 |
| Trifluorophenol | 0·7 | 96·3 | 57·5 |
| Tetrafluorophenol | — | 123·1 | 60·3 |
| Pentafluorophenol | 0·3 | 236·8 | 63·2 |

Ferguson (1939) showed with a homologous series of normal primary alcohols that a nearly parallel relation existed between solubilities, toxic concentrations, concentrations reducing the surface tension of water to 50 $mNm^{-1}$, vapour pressures and partition coefficients. Fig. 6 summarizes our results for phenol and the fluorophenols.

An analysis of variance and a test for parallelism on the four regressions in Fig. 6 gave $F^3_{20} = 8{\cdot}57$. The tabulated value of $F^3_{20}$ ($P' = 0{\cdot}05$) is 3·10; since the tabulated value is much less than that calculated, the lines may not be regarded as parallel. A similar calculation on the slopes of the regression lines for toxicity, partition coefficients and surface tension reduction, gave $F^2_{15} = 3{\cdot}28$ (tabulated value = 3·68; $P' = 0{\cdot}05$). The slopes of the regressions relating these three properties to the number of fluorine substituents are therefore not significantly different.

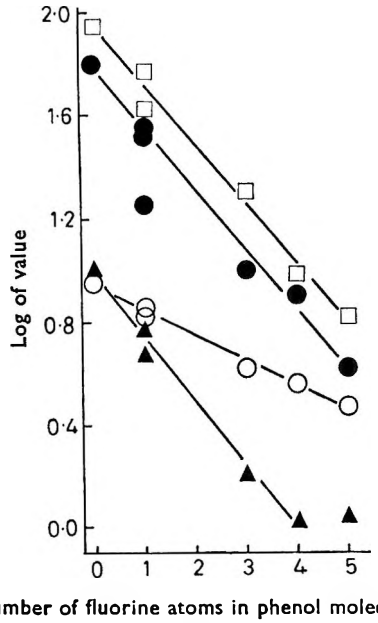


FIG. 6. Correlation of toxic concentrations (99·9% mortality in 50 min) and physical properties of fluorophenols with the number of fluorine atoms substituted in the phenol molecule. □ = molar toxic concentration, × $10^3$. ● = 1/oleyl alcohol-water partition coefficient, × $10^3$. ○ = molar solubility, × 10. ▲ = molar concentration to reduce surface tension of water to 60 $mNm^{-1}$ (dynes/cm), × $10^2$.

## DISCUSSION

Chauhan & Walters (1967) showed that the log survivor-time curves for *Penicillium notatum* spores exposed to phenols became increasingly concave to the axes as the aqueous solubilities of the compounds decreased and, therefore, the oil-water partition coefficients increased. A similar trend (for example, Fig. 1) has been obtained with the fluorophenols as the series is ascended from the more water soluble unsubstituted phenol (Table 2) to the more lipid soluble pentafluorophenol (Table 3 and Fig. 5).

The concavity of the time-survivor curves may explain why tetra- and pentafluorophenol are more active when compared with phenol at the 99·9% mortality level than by the Rideal-Walker test (Table 1). Thus, the initial reaction rate is rapid for the higher fluorophenols and decreases with increase in mortality level. Therefore, a given concentration will, relative to phenol, take longer to reach the lower survivor levels of the Rideal-Walker test than to attain a 99·9% kill.

The log survivor-time curves for the lowest concentrations of tetra- and pentafluorophenol are concave to the axes to about the 5% survivor level; the rate of kill then increases and they become convex (Fig. 1). This apparent change in the rate of reaction is unlikely to be due to adsorption or penetration phenomena since, in each case, the second phase of rapid mortality does not commence until after about 60 min exposure. It is probable that the shape of the curves is determined by the distribution of resistance in the bacterial population.

The thermodynamic activities of equitoxic solutions of the fluorophenols (Table 2), decrease with increasing fluorine substitution and, therefore, with increasing molecular

weight of the compounds. A similar relation was found by James, Loveday & Plummer (1964) with *p*-halogenated phenols. These authors also investigated the series: phenol, *p*-cresol, *p*-ethylphenol and *p*-n-propylphenol. The activities remained almost constant as did those of the phenols examined by Allawala & Riegelman (1954). Both of these trends differ from that originally described by Ferguson (1939) and considered normal by Sexton (1963), namely that activities rise as a homologous series is ascended. Sexton (1963) considers this rise to be due to departure from ideality in the physical properties of high molecular weight substances. The comparatively lower molecular weights of the fluorophenols may be the reason why a similar trend was not found.

The partition coefficients between oleyl alcohol and water increase with toxicity, but those between cyclohexane and water do not (Fig. 5 and Table 3). These results substantiate the findings of Burton, Clarke & Gray (1964); only organic solvents which form hydrogen bonds with phenols should be used for partition coefficient measurements when the results are to be compared with bactericidal activities. The partition coefficients of the fluorophenols between oleyl alcohol and water represent their bulk phase distribution, and hydrogen bonding which occurs between the phenol and the lipid may assist transfer of the phenol molecule from the aqueous to the organic phase (Burton & others, 1964). Such a mechanism could occur at or above the cell membranes of organisms where a large amount of lipid is located (Gilby & Few, 1957; McQuillen, 1960; Martin, 1963). Correlations between increasing lipid solubility of phenols and both their uptake by *E. coli* and their bactericidal activities have been demonstrated by Judis (1964). It is possible that hydrogen bonding between phenols and the lipoprotein and lipopolysaccharide of the cell wall and membrane is part of the mechanism involved in the disruption of the permeability barriers of the cell which results in lysis and death.

The lipid content of Gram-negative cells is much greater than in Gram-positive organisms and a high lipid content has been implicated in resistance to disinfectants (Chaplin, 1952; Truby & Bennett, 1966; Hugo, 1967; Hugo & Franklin, 1968). It may be that the inner layers of the lipoprotein of lipid rich membranes are not so readily damaged and thereby permeability barriers are maintained.

Equitoxic solutions of the bactericides have approximately the same surface tension (Table 3) in agreement with the results of Traube & Somogyi (1921), Frobisher (1927) and Zissmann (1954, 1957). Such correlation is found only with surfactant solutions which are antibacterial. Thus James (1965) observes that reduction of surface tension is not in itself bactericidal, since non-ionic surface-active agents are non-toxic (Baker, Harrison & Miller, 1941; Hotchkiss, 1946).

The regressions relating log molar toxic concentration, partitioning (plotted as the reciprocal of the partition coefficient so as to obtain a line of negative slope) and surface tension reduction, to the number of fluorine substituents in the phenol molecule are parallel (Fig. 6). However, although there is a linear correlation between log molar solubility and log molar toxic concentration (Fig. 4), the reduction in solubility is not paralleled by the increase in toxicity (Fig. 6). It would appear that a better prediction of bactericidal activity may be obtained from partitioning data than from solubility determinations. Similar conclusions have been drawn by Hess & Speiser (1959) and McCulloch & Stock (1966).

Thus although the principle of Ferguson (1939) is of great value in determining whether toxic action is of a chemical or physical nature, i.e. whether death is due to

interference with cell biochemistry or results from non-specific structural damage, deviations from the ideal state of solutions and approximations produced by derivation of thermodynamic activities from solubility data, may result in better correlations of toxicity being obtained with some physico-chemical property other than solubility.

*Acknowledgements*

We thank Mr. B. Warburton for assistance with the surface tension determinations. One of us (R. J. P.) is grateful to the Science Research Council for the award of a Research Studentship during part of the work.

## REFERENCES

ALLAWALA, N. A. & RIEGELMAN, S. (1954). *J. pharm. Sci.*, **43**, 93–97.
BAKER, Z., HARRISON, R. W. & MILLER, B. F. (1941). *J. exp. Med.*, **73**, 249–271.
BURTON, D. E., CLARKE, K. & GRAY, G. W. (1964). *J. chem. Soc.*, 1314–1318.
CHAPLIN, C. E. (1952). *J. Bact.*, **63**, 453–458.
CHAUHAN, N. M. & WALTERS, V. (1967). *J. Pharm. Pharmac.*, **19**, 735–739.
FERGUSON, J. (1939). *Proc. R. Soc.*, **127B**, 387–404.
FROBISHER, M. (1927). *J. Bact.*, **13**, 163–182.
GILBY, A. R. & FEW, A. V. (1957). *Proc. 2nd Int. Congr. Surf. Activ.*, **IV**, 262–270.
HESS, H. & SPEISER, P. (1959). *J. Pharm. Pharmac.*, **11**, 694–702.
HOTCHKISS, R. D. (1946). *Ann. N.Y. Acad. Sci.*, **46**, 479–93.
HUGO, W. B. (1967). *J. appl. Bact.*, **30**, 17–50.
HUGO, W. B. & FRANKLIN, I. (1968). *J. gen. Microbiol.*, **52**, 365–373.
JAMES, A. M. (1965). in *Surface Activity and the Microbial Cell*, S.C.I. Monograph No. 19, 3–22, London: Society of Chemical Industry.
JAMES, A. M., LOVEDAY, D. E. E. & PLUMMER, D. T. (1964). *Biochim. biophys. Acta*, **79**, 351–363.
JUDIS, J. (1964). *J. pharm. Sci.*, **53**, 196–201.
KLARMANN, E., SHTERNOV, V. A. & VON WOWERN, J. (1929). *J. Bact.*, **17**, 423–442.
MARTIN, H. H. (1963). *J. theoret. Biol.*, **5**, 1–34.
MCCULLOCH, A. C. & STOCK, B. H. (1966). *Australas. J. Pharm.*, **48**, S14–S16.
MCQUILLEN, K. (1960). *The Bacteria*, **I**, p. 300, London: Academic Press.
ORDAL, E. J. (1941). *Proc. Soc. exp. Biol. Med.*, **47**, 387–389.
PADDAY, J. F. (1957). *Proc. 2nd Int. Congr. Surf. Activ.*, **I**, 1–6.
PINNEY, R. J. & WALTERS, V. (1967). *J. Pharm. Pharmac.*, **19**, *Suppl.*, 177S–182S.
REESE, D. R., IRWIN, G. M., DITTERT, L. W., CHONG, C. W. & SWINTOSKY, J. V. (1964). *J. pharm. Sci.*, **53**, 591–597.
SEXTON, W. A. (1963). *Chemical Constitution and Biological Activity*, 3rd edn, London: Spon.
SYKES, G. (1965). *Disinfection and Sterilization*, 2nd edn, London: Spon.
TRAUBE, J. & SOMOGYI, R. (1921). *Biochem. Z.*, **120**, 90–99.
TRUBY, C. P. & BENNETT, E. O. (1966). *Appl. Microbiol.*, **14**, 769–773.
WARBURTON, B. (1966). in *The Chemistry and Rheology of Water Soluble Gums and Colloids*, S.C.I. Monograph No. 24, 118–130, London: Society of Chemical Industry.
WOLF, P. A. & WESTVEER, W. M. (1952). *Archs Biochem. Biophys.*, **40**, 306–309.
ZISSMANN, E. (1954). *C. r. hebd. Séanc. Acad. Sci. Paris*, **238**, 1843–1845.
ZISSMANN, E. (1957). *Ibid.*, **245**, 237–240.

*J. Pharm. Pharmac.*, 1969, **21**, 423–433 *Received January* 1, 1969

# Antituberculosis activity of some nitrofuran derivatives

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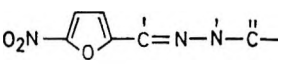
A series of 4,6-disubstituted 2-(5-nitrofurfurylidenehydrazino)-1,3,5-triazines has been synthesized. Some of these compounds possessed good antibacterial activity against *Mycobacterium tuberculosis in vitro* and moderate activity *in vivo* (the activity *in vivo* was observed only with high dosages compared with compounds in clinical use and the results were also irregular). The most active compounds are triazinyl-hydrazones of 5-nitro-2-furaldehyde in which the triazine ring is substituted with two amino-groups, at least one of which is an (α-branched chain alkyl)- or cycloalkylamine. Structure- anti-tuberculosis activity relations for these compounds are discussed.

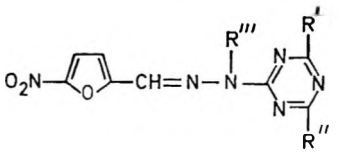
Many hundreds of nitrofurans have been reported in the literature (Paul & Paul, 1964; Ellis & West, 1967) but so far no nitrofurans have been claimed to have anti-tuberculosis activity *in vivo* (Schnitzer, 1964; Ellis & West, 1967). We now wish to report such anti-tuberculosis activity in a series of diamino-1,3,5-trizainylhydrazones of 5-nitro-2-furaldehyde.

Dodd, Cramer, & Ward (1950) noted that the basic structure I usually conferred *in vivo* antibacterial activity. It occurred to us that triazinylhydrazones of 5-nitro-2-furaldehyde would conform to such structure requirements. Accordingly, a number



(I)

of compounds of Type II and some simple analogues were synthesized and we now report the unusual pharmacological and microbiological properties of a wide range of triazinylhydrazones of 5-nitro-2-furaldehyde and other aldehydes.‡



(II)

In the first instance the readily accessible 2-chloro-4,6-di(substituted amino)-1,3,5-triazines (Pearlman & Banks, 1948; Thurston, Dudley & others, 1951) were reacted with three molecular proportions of hydrazine hydrate in refluxing ethanol to give the corresponding new di(substituted amino)triazinylhydrazines which, after isolation by

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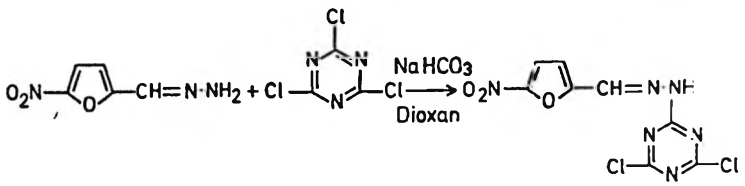
‡ During the course of this work Hirao & Kato (1964) described the preparation of several nitrofurans of this type (cpds 22, 26, 28, 45, 51 and 55).

one of two techniques described, readily condensed with 5-nitro-2-furaldehyde to give compounds of Type III (see Table 1). The analogous aldehydes: 2-furaldehyde, β-(5-nitrofur-2-yl)acrylaldehyde, 5-nitrothiophen-2-carbaldehyde, and 5-nitropyrrole-2-carbaldehyde, gave the corresponding hydrazones of Type V (see Table 3).

In a similar manner methylhydrazine and hydroxyethylhydrazine gave the corresponding substituted diaminotriazinylhydrazines which likewise condensed with 5-nitro-2-furaldehyde to give compounds of Type IV (see Table 2).

Investigation of triazinylhydrazones with substituents other than amino-groups on the triazine ring was made difficult by the inaccessibility of many of the required intermediates. However it was found that 5-nitro-2-furaldehyde hydrazone (ICI, 1959) reacts with cyanuric chloride to give the stable dichloro-derivative VI. Using




(VI)

the method of Senier (1886) this dichloro-derivative was treated with hot glacial acetic acid to give the corresponding dihydroxy-compound. Reaction of the dichloro-derivative VI with sodium methoxide gave the dimethoxy-derivative. The chlorine atoms could also be replaced by amino-groups and a combination of such reactions led to successive replacement of the chlorine atoms in VI to give mixed chloro-amino- or hydroxy-amino-triazinylhydrazones. The di-xylyl derivative was prepared in a similar manner to the diaminotriazinylhydrazines from the 2-chloro-4,6-di(2,4-xylyl)-1,3,5-triazine described by CIBA (1961). The trichloromethyl group in 2-amino-4-methyl-6-trichloromethyl-1,3,5-triazine (Kreutzberger, 1957) was replaced by the hydrazino-group by reaction with hydrazine; subsequent condensation with 5-nitro-2-furaldehyde gave the desired product. Typical preparative details are given in the experimental section and the melting point and analysis of the compounds are listed in Tables 1, 2, 3, and 4.

## EXPERIMENTAL

### *Chemical*

All melting points are uncorrected.

2-*Hydrazino*-4,6-*dipiperidino*-1,3,5-*triazine*. 2-Chloro-4,6-dipiperidino-1,3,5-triazine (Pearlman & Banks, 1948) (17·6 g) was dissolved in ethanol (50 ml) under reflux and then treated with hydrazine hydrate (100%) (9·4 ml). The mixture was refluxed for 1 h during which time the product deposited. On completion of the reaction the liquor was cooled and the product was collected by filtration and washed well with water. The solid was recrystallized from a mixture of light petroleum (b.p. 100–120°) and ethanol (10:1) to give the hydrazine (12·4 g), m.p. 130°. (Found: C, 56·1; H, 8·3; N, 35·3. $C_{13}H_{25}N_7$ Requires: C, 56·3; H, 8·4; N, 35·35%).

2-*Ethylamino*-4-*hydrazino*-6-*isopropylamino*-1,3,5-*triazine*. 2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine (21·6 g) was suspended in ethanol (150 ml) under reflux and then treated with hydrazine hydrate (100%) (15 ml). The mixture was refluxed for 1 h. Ethanol (50 ml) was removed by distillation and water (100 ml) was

added to the residue. The aqueous ethanolic solution of the residue was extracted with chloroform (2 × 50 ml, 2 × 25 ml) and the chloroform extract was washed with water (25 ml) and dried over anhydrous sodium sulphate. Removal of the chloroform gave the hydrazine (21·1 g) as a syrup which could not be crystallized and which was reacted directly to give the hydrazone.

2-*Ethylamino*-4-*isopropylamino*-6-(5-*nitrofurfurylidenehydrazino*)-1,3,5-*triazine*. Crude 2-ethylamino-4-hydrazino-6-isopropylamino-1,3,5-triazine (21·1 g) was dissolved in ethanol (100 ml) and treated with 5-nitro-2-furaldehyde (14·1 g). The mixture was refluxed for ½ h and then cooled to 5°. The yellow product which precipitated was collected by filtration and washed with ethanol (20 ml). The solid was recrystallized from nitromethane (125 ml) and then ethanol (250 ml) to give the hydrazone (12 g), m.p. 199–201° (Found: C, 46·7; H, 5·4; N, 33·6. $C_{13}H_{18}N_8O_3$ Requires: C, 46·7; H, 5·4; N, 33·5%).

2,4-*Dichloro*-6-(5-*nitrofurfurylidenehydrazino*)-1,3,5-*triazine*. A solution of cyanuric chloride (6·2 g) in dioxan (50 ml) was prepared and to this vigorously stirred solution was added simultaneously a solution of 5-nitro-2-furaldehyde hydrazone (ICI, 1959) in dioxan (150 ml) at 30° and a solution of sodium bicarbonate (2·8) in water (50 ml). The addition was completed in 15 min and the mixture was then stirred a further 2 h. The yellow product was collected by filtration and washed with aqueous dioxan. The solid was dried to give the crude hydrazone (7·3 g), which was then extracted with nitroethane in a Soxhlet apparatus. The nitroethane extract deposited crystals of the pure hydrazone, m.p. > 300° (Found: C, 31·6; H, 1·3; Cl, 23·5, $C_8H_4Cl_2N_6O_3$ Requires: C, 31·7; H, 1·3; Cl, 23·4%).

2,4-*Dihydroxy*-6-(5-*nitrofurfurylidenehydrazino*)-1,3,5-*triazine*. 2,4-Dichloro-6-(5-nitrofurfurylidenehydrazino)-1,3,5-triazine (5 g) was extracted with glacial acetic acid (500 ml) in a sintered glass Soxhlet apparatus for 24 h. The extract was cooled slightly and the decomposition product removed by filtration. On further cooling the filtrate deposited the product which was collected by filtration and dried. The dry material (2·3 g) was extracted with nitromethane for 8 h in a Soxhlet apparatus and on completion of the extraction the nitromethane extract deposited yellow crystals of the hydrazone (0·4 g), m.p. > 300° (Found: C, 36·1; H, 2·45; N, 31·7. $C_8H_6N_6O_5$ Requires: C, 36·1; H, 2·3; N, 31·6).

2,4-*Dimethoxy*-6-(5-*nitrofurfurylidenehydrazino*)-1,3,5-*triazine*. 2,4-Dichloro-6-(5-nitrofurfurylidenehydrazino)-1,3,5-triazine (20 g) was suspended in methanol (350 ml). To this suspension a solution of sodium methoxide, prepared from sodium (30·6 g) and dry methanol (650 ml), was added dropwise over 1½ h. On completion of the addition the solution was allowed to stand at room temperature for 4 h. The solid which precipitated was discarded and the resulting clear liquor was cooled to give a yellow product. Evaporation of the mother liquor gave further crops of the product. The solid was recrystallized from nitroethane to give the hydrazone (5 g), m.p. 260° (dec.) (Found: C, 40·65; H, 3·4; N, 28·7. $C_{10}H_{10}N_6O_5$ Requires: C, 40·8; H, 3·4; N, 28·6%).

2-*Chloro*-4-*diethylamino*-6-(5-*nitrofurfurylidenehydrazino*)-1,3,5-*triazine*. 2,4-Dichloro-6-(5-nitrofurfurylidenehydrazino)-1,3,5-triazine (6·1 g) was dissolved in dimethyl sulphoxide (100 ml) at 50°. To this solution at 50° ± 2°, a solution of diethylamine (3·0 g) in chloroform (10 ml) was added dropwise during ½ h. On completion of the addition the solution was allowed to stand at room temperature for 1 h and was then poured into water (200 ml) with stirring. The yellow product which deposited was

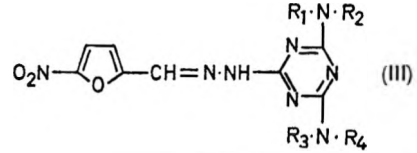


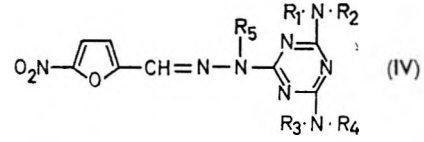

Table 1. *Diaminotriazinylhydrazones of 5-nitro-2-furaldehyde*

| Cpd no. | $R_1$ | $R_2$ | $R_3$ | $R_4$ | m.p. °C | Formula | Found C | Found H | Found N | Found S | Required C | Required H | Required N | Required S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | H | H | H | H | *a* >200 (dec.) | $C_8H_8N_8O_3 \cdot C_4H_4O_4$ | 37·8 | 3·45 | 29·5 | | 37·9 | 3·2 | 29·5 | |
| 2 | Me | H | H | H | *b* 221 | $C_9H_{10}N_8O_3 \cdot \frac{1}{2}H_2SO_4 \cdot \frac{3}{4}H_2O$ | 31·7 | 4·0 | 32·7 | 4·65 | 31·7 | 3·8 | 32·9 | 4·70 |
| 3 | Et | H | H | H | 244–6 (dec.) | $C_{10}H_{12}N_8O_3$ | 41·2 | 4·4 | | | 41·1 | 4·1 | | |
| 4 | $Pr^i$ | H | H | H | 237–9 (dec.) | $C_{11}H_{14}N_8O_3 \cdot \frac{1}{2}H_2O$ | 42·1 | 4·7 | | | 41·9 | 4·8 | | |
| 5 | $C_3H_5$ | H | H | H | 225 (dec.) | $C_{11}H_{12}N_8O_3$ | 43·3 | 4·2 | 36·7 | | 43·4 | 4·0 | 36·8 | |
| 6 | $Bu^s$ | H | H | H | 164 | $C_{12}H_{16}N_8O_3$ | 44·95 | 5·15 | 34·7 | | 45·0 | 5·0 | 35·0 | |
| 7 | $Bu^t$ | H | H | H | 199–200 | $C_{12}H_{16}N_8O_3$ | 45·3 | 5·05 | 35·0 | | 45·0 | 5·0 | 35·0 | |
| 8 | $C_5H_9$*c* | H | H | H | 218 | $C_{13}H_{16}N_8O_3 \cdot \frac{1}{2}H_2O$ | 45·6 | 5·1 | 33·1 | | 45·7 | 5·0 | 32·8 | |
| 9 | s-$C_5H_{11}$ | H | H | H | 138–140 (dec.) | $C_{13}H_{18}N_8O_3$ | 46·8 | 5·6 | 33·8 | | 46·7 | 5·4 | 33·5 | |
| 10 | Me·$CH_2$·CH(Et) | H | H | H | 167 (dec.) | $C_{13}H_{18}N_8O_3 \cdot \frac{1}{2}H_2O$ | 45·3 | 5·6 | 32·6 | | 45·5 | 5·6 | 32·6 | |
| 11 | $C_6H_{11}$*d* | H | H | H | 218–222 (dec.) | $C_{14}H_{18}N_8O_3$ | 48·4 | 5·45 | 32·4 | | 48·5 | 5·2 | 32·3 | |
| 12 | Ph | H | H | H | 282 | $C_{14}H_{12}N_8O_3$ | 49·4 | 3·7 | 33·1 | | 49·4 | 3·55 | 32·9 | |
| 13 | *o*-MeO·$C_6H_4$ | H | H | H | 268–9 (dec.) | $C_{15}H_{14}N_8O_4 \cdot H_2O$ | 48·1 | 4·2 | 30·1 | | 48·4 | 4·3 | 30·1 | |
| 14 | *m*-$CF_3$·$C_6H_4$ | H | H | H | 246 (dec.) | $C_{15}H_{11}F_3N_8O_3$ | 44·4 | 2·6 | 27·3 | | 44·1 | 2·7 | 27·5 | |
| 15 | Ph·$CH_2$ | H | H | H | 215 | $C_{15}H_{14}N_8O_3$ | 50·7 | 4·3 | 31·5 | | 50·8 | 4·0 | 31·6 | |
| 16 | $Pr^i$ | H | Me | H | 203–5 (dec.) | $C_{12}H_{16}N_8O_3$ | 45·0 | 5·2 | 34·9 | | 45·0 | 5·0 | 35·0 | |
| 17 | $Pr^i$ | H | Et | H | | | | | | | | | | |
| 18 | $Pr^i$ | H | $Pr^i$ | H | 220–2 | $C_{14}H_{20}N_8O_3$ | 48·5 | 5·9 | 32·45 | | 48·25 | 5·8 | 32·2 | |
| 19 | $Pr^i$ | H | $Bu^s$ | H | 207 9 (dec.) | $C_{15}H_{22}N_8O_3$ | 49·5 | 6·1 | 30·85 | | 49·7 | 6·1 | 30·9 | |
| 20 | $Pr^i$ | H | Ph | H | 186–8 (dec.) | $C_{17}H_{18}N_8O_3$ | 53·55 | 5·0 | 29·1 | | 53·4 | 4·7 | 29·3 | |
| 21 | Et | Et | H | H | 254 (dec.) | $C_{12}H_{16}N_8O_3$ | 45·0 | 4·9 | | | 45·0 | 5·0 | | |
| 22 | Et | H | Et | H | 220–2 | $C_{12}H_{16}N_8O_3$ | 44·85 | 5·2 | | | 45·0 | 5·0 | | |
| 23 | Et | Et | Et | H | 185–7 | $C_{14}H_{20}N_8O_3$ | 48·3 | 5·8 | | | 48·25 | 5·8 | | |
| 24 | Et | Et | Et | Et | 111–2 | $C_{16}H_{24}N_8O_3$ | 51·3 | 6·55 | | | 51·05 | 6·4 | | |
| 25 | Me | Me | H | H | 267–9 (dec.) | $C_{10}H_{12}N_8O_3$ | 41·1 | 4·15 | 38·1 | | 41·1 | 4·1 | 38·35 | |
| 26 | Me | H | Me | H | 249–250 | $C_{10}H_{12}N_8O_3$ | 41·0 | 4·3 | 38·1 | | 41·1 | 4·1 | 38·25 | |
| 27 | Me | Me | Me | H | 250–2 (dec.) | $C_{11}H_{14}N_8O_3$ | 43·1 | 4·7 | | | 43·1 | 4·6 | | |
| 28 | Me | Me | Me | Me | 201–2 | $C_{12}H_{16}N_8O_3$ | 44·9 | 4·9 | 35·2 | | 45·0 | 5·0 | 35·0 | |
| 29 | Me | H | Et | H | 234–7 (dec.) | $C_{11}H_{14}N_8O_3$ | 43·2 | 4·55 | | | 43·1 | 4·6 | | |
| 30 | Me | H | $Pr^n$ | H | *b* 178–185 | $C_{12}H_{16}N_8O_3 \cdot \frac{1}{2}H_2SO_4 \cdot \frac{1}{2}H_2O$ | 37·9 | 5·1 | 29·7 | | 38·1 | 4·8 | 29·6 | |
| 31 | Me | H | $Bu^n$ | H | 168–170 | $C_{13}H_{18}N_8O_3$ | 46·5 | 5·3 | 33·7 | | 46·7 | 5·4 | 33·5 | |
| 32 | Me | H | $Bu^s$ | H | 171–2 | $C_{13}H_{18}N_8O_3$ | 46·6 | 5·6 | 33·6 | | 46·7 | 5·4 | 33·5 | |
| 33 | Me | H | $Bu^t$ | H | 219–222 (dec.) | $C_{13}H_{18}N_8O_3$ | 46·4 | 5·4 | 33·6 | | 46·7 | 5·4 | 33·5 | |

Table 1—*continued*

| Cpd no. | $R_1$ | $R_2$ | $R_3$ | $R_4$ | | m.p. | °C | Formula | Found C | Found H | Found N | Found S | Required C | Required H | Required N | Required S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 34 | Me | H | $C_5H_9$*c* | H | | 136 | (dec.) | $C_{14}H_{18}N_8O_3$ | 48·5 | 5·3 | 32·55 | | 48·5 | 5·2 | 32·3 | |
| 35 | Me | H | s-$C_8H_{17}$ | H | | 153–5 | | $C_{17}H_{26}N_8O_3$ | 52·2 | 6·65 | | | 52·3 | 6·7 | | |
| 36 | Me | H | Ph | H | | 236 | (dec.) | $C_{15}H_{14}N_8O_3$ | 50·8 | 4·1 | 31·5 | | 50·8 | 4·0 | 31·6 | |
| 37 | Et | H | $C_3H_6OMe$ | H | | 179–182 | | $C_{14}H_{20}N_8H_4\cdot\frac{1}{2}H_2SO_4\cdot 2H_2O$ | 37·7 | 5·7 | | 3·75 | 37·5 | 5·6 | | 3·6 |
| 38 | $Pr^i$ | H | $C_3H_5$ | H | | 201–2 | | $C_{14}H_{18}N_8O_3$ | 48·7 | 5·5 | 32·2 | | 48·5 | 5·2 | 32·4 | |
| 39 | $Pr^n$ | H | $C_2H_4OH$ | H | | 152–4 | | $C_{13}H_{18}N_8O_4$ | 44·4 | 5·2 | | | 44·55 | 5·2 | | |
| 40 | $C_3H_5$ | H | $C_3H_5$ | H | | 189–190 | (dec.) | $C_{14}H_{18}N_8O_3$ | 48·9 | 4·65 | 32·5 | | 48·8 | 4·7 | 32·6 | |
| 41 | $Pr^i$ | H | $C_3H_6OMe$ | H | | 106–7 | (dec.) | $C_{15}H_{22}N_8O_4\cdot\frac{1}{2}H_2O$ | 46·7 | 6·0 | 28·9 | | 46·5 | 6·0 | 29·0 | |
| 42 | $Bu^s$ | H | $Bu^s$ | H | *b* | 185–7 | | $C_{16}H_{24}N_8O_3\cdot\frac{1}{2}H_2SO_4\cdot 2H_2O$ | 41·5 | 6·4 | 24·2 | 3·6 | 41·6 | 6·6 | 24·3 | 3·4 |
| 43 | $Bu^t$ | H | $Bu^t$ | H | | 269–271 | (dec.) | $C_{16}H_{24}N_8O_3$ | 51·3 | 6·4 | 29·4 | | 51·05 | 6·4 | 29·8 | |
| 44 | $C_{12}H_{25}$ | H | $C_{12}H_{25}$ | H | | 145–6 | | $C_{32}H_{56}N_8O_3\cdot\frac{1}{4}H_2O$ | 63·8 | 9·2 | 18·3 | | 63·5 | 9·4 | 18·5 | |
| 45 | $C_5H_9$*c* | H | $C_5H_9$*c* | H | *b* | 259 | (dec.) | $C_{18}H_{24}N_8O_3\cdot\frac{1}{2}H_2SO_4\cdot H_2O$ | 46·2 | 5·85 | 24·0 | 3·4 | 46·25 | 5·8 | 24·0 | 3·4 |
| 46 | $C_6H_{11}$*d* | H | $C_6H_{11}$*d* | H | | 218–220 | (dec.) | $C_{20}H_{28}N_8O_3$ | 56·2 | 6·7 | 25·9 | | 56·1 | 6·6 | 26·15 | |
| 47 | Ph | H | Ph | H | | 255–8 | (dec.) | $C_{20}H_{16}N_8O_3$ | 57·4 | 3·9 | | | 57·6 | 3·9 | | |
| 48 | $Ph\cdot CH_2$ | H | $PhCH_2$ | H | | 203–4 | | $C_{22}H_{20}N_8O_3$ | 59·2 | 4·5 | | | 59·4 | 4·5 | | |
| 49 | H | H | $CH_2\cdot CH_2\cdot CH_2\cdot CH_2$ | | | 282 | (dec.) | $C_{12}H_{14}N_8O_3$ | 45·5 | 4·6 | 35·1 | | 45·3 | 4·4 | 35·2 | |
| 50 | H | H | $CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2$ | | | 270 | (dec.) | $C_{13}H_{16}N_8O_3$ | 46·8 | 5·1 | 33·8 | | 47·0 | 4·85 | 33·7 | |
| 51 | Me | H | Et | Et | | 220–1 | (dec.) | $C_{13}H_{18}N_8O_3$ | 46·8 | 5·5 | | | 46·7 | 5·4 | | |
| 52 | $Pr^i$ | H | Me | Me | | 194–5 | | $C_{13}H_{18}N_8O_3$ | 46·7 | 5·5 | 33·45 | | 46·7 | 5·4 | 33·5 | |
| 53 | $Pr^i$ | H | Et | Et | | 172–4 | | $C_{15}H_{22}N_8O_3$ | 49·9 | 6·0 | 31·2 | | 49·7 | 6·1 | 30·9 | |
| 54 | Et | Et | $CH_2\cdot CH_2\cdot CH_2\cdot CH_2$ | | | 188–200 | (dec.) | $C_{16}H_{22}N_8O_3$ | 51·3 | 6·2 | 30·0 | | 51·3 | 5·9 | 29·9 | |
| 55 | $CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot$ | | $CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2$ | | | 218 | (dec.) | $C_{18}H_{24}N_8O_3$ | 54·2 | 6·2 | 27·9 | | 54·0 | 6·0 | 28·0 | |
| 56 | $CH_2\cdot CH_2\cdot O\cdot CH_2\cdot CH_2$ | | $CH_2\cdot CH_2\cdot O\cdot CH_2\cdot CH_2$ | | | 262 | (dec.) | $C_{16}H_{20}N_8O_5\cdot\frac{1}{2}C_2H_5OH$ | 47·9 | 5·5 | 26·3 | | 47·8 | 5·4 | 26·3 | |
| 57 | $HO\cdot[CH_2]_2$ | $HO\cdot[CH_2]_2$ | $HO\cdot[CH_2]_2$ | $HO\cdot[CH_2]_2$ | | 190–2 | | $C_{16}H_{24}N_8O_7\cdot H_2O$ | 42·2 | 5·7 | 24·3 | | 41·9 | 5·7 | 24·45 | |

*a* Maleate. *b* Sulphate. *c* Cyclopentyl. *d* Cyclohexyl.




(IV)

Table 2. *Substituted diaminotriazinylhydrazones of 5-nitro-2-furaldehyde*

| Cpd no. | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | m.p. °C. | Formula | Found C | Found H | Found N | Found S | Required C | Required H | Required N | Required S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 58 | Me | H | Me | H | Me | *b* 248–9 | $C_{11}H_{14}N_8O_3 \cdot \frac{1}{2}H_2SO_4 \cdot \frac{1}{2}H_2O$ | 36·6 | 4·5 | 31·2 | 4·5 | 36·7 | 4·3 | 31·1 | 4·45 |
| 59 | Et | H | Pr$^i$ | H | Me | *b* 271 (dec.) | $C_{14}H_{20}N_8O_3 \cdot H_2SO_4$ | 37·8 | 5·1 | 25·2 | 7·2 | 37·7 | 5·0 | 25·2 | 7·2 |
| 60 | Pr$^i$ | H | Pr$^i$ | H | Me | 146–7 | $C_{15}H_{22}N_8O_3$ | 49·7 | 6·1 | 30·9 | | 49·6 | 6·3 | 30·8 | |
| 61 | $C_{12}H_{25}$ | H | $C_{12}H_{25}$ | H | Me | *b* 237 | $C_{33}H_{56}N_8O_3 \cdot H_2SO_4$ | 55·7 | 8·2 | 15·7 | 4·2 | 55·8 | 8·2 | 15·8 | 4·5 |
| 62 | Ph | H | Ph | H | Me | 235 | $C_{21}H_{18}N_8O_3$ | 58·4 | 4·1 | 25·8 | | 58·6 | 4·2 | 26·0 | |
| 63 | MeO·$[CH_2]_3$ | H | $C_6H_{11}$*d* | H | Me | *b* 252 (dec.) | $C_{19}H_{28}N_8O_3 \cdot H_2SO_4 \cdot H_2O$ | 42·9 | 5·7 | 20·9 | 5·8 | 42·85 | 5·95 | 21·0 | 6·0 |
| 64 | $C_{12}H_{25}$ | H | HO·$[CH_2]_2$ | HO·$[CH_2]_2$ | Me | 115–7 | $C_{25}H_{42}N_8O_5$ | 56·2 | 8·1 | 20·9 | | 56·3 | 7·75 | 21·0 | |
| 65 | Ph·$CH_2$ | H | $CH_2 \cdot CH_2 \cdot O \cdot CH_2 \cdot CH_2$ | | Me | 178–180 | $C_{20}H_{22}N_8O_4$ | 54·7 | 5·2 | 25·6 | | 54·8 | 5·1 | 25·6 | |
| 66 | Me | Me | Me | Me | Me | 211–2 | $C_{13}H_{18}N_8O_3$ | 46·9 | 5·65 | | | 46·7 | 5·4 | | |
| 67 | Et | Et | Et | Et | Me | 138–140 | $C_{17}H_{26}N_8O_3$ | 52·1 | 6·6 | 29·0 | | 52·3 | 6·7 | 28·7 | |
| 68 | $CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2$ | | $CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2$ | | Me | 232 | $C_{19}H_{26}N_8O_3$ | 54·9 | 6·45 | 26·8 | | 55·05 | 6·3 | 27·0 | |
| 69 | Et | H | Et | H | HO·$[CH_2]_2$ | 211–2 (dec.) | $C_{14}H_{20}N_8O_4$ | 46·1 | 5·5 | 30·7 | | 46·1 | 5·5 | 30·75 | |
| 70 | Et | H | Pr$^i$ | H | HO·$[CH_2]_2$ | 187–8 | $C_{15}H_{22}N_8O_4$ | 47·8 | 6·1 | 29·35 | | 47·6 | 5·9 | 29·6 | |
| 71 | Et | MeCO | Et | Me·CO | Me·CO·O·$[CH_2]_2$ | 191–2 | $C_{20}H_{26}N_8O_7$ | 49·2 | 5·1 | 22·9 | | 49·0 | 5·3 | 22·85 | |

*b* sulphate. *d* cyclohexyl.

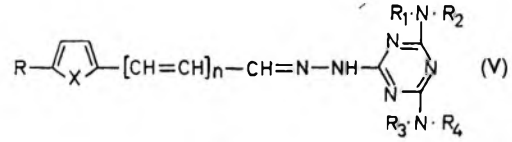



Table 3. *Diaminotriazinylhydrazones of various other aldehydes*

| Cpd no. | R | X | n | $R_1$ | $R_2$ | $R_3$ | $R_4$ | m.p. | °C. | Formula | Found C | Found H | Found N | Found S | Required C | Required H | Required N | Required S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 72 | $NO_2$ | O | 1 | Me | H | $Pr^i$ | H | *b* 258 | (dec.) | $C_{14}H_{18}N_8O_3 \cdot H_2SO_4 \cdot H_2O$ | 36·5 | 5·0 | 24·4 | | 36·4 | 4·8 | 24·2 | |
| 73 | $NO_2$ | O | 1 | Me | Me | Me | Me | 244–6 | (dec.) | $C_{14}H_{18}N_8O_3$ | 48·7 | 5·1 | | | 48·5 | 5·2 | | |
| 74 | H | O | 0 | Me | H | $Pr^i$ | H | *e* 170–4 | (dec.) | $C_{12}H_{17}N_7O \cdot HCl \cdot H_2O$ | 43·9 | 6·2 | 29·7 | | 43·7 | 6·1 | 29·7 | |
| 75 | H | O | 0 | Et | H | Et | H | 100–112 | | $C_{12}H_{17}N_7O$ | 52·2 | 6·3 | | | 52·35 | 6·2 | | |
| 76 | H | O | 0 | Me | Me | Me | Me | 210–1 | | $C_{12}H_{17}N_7O$ | 52·3 | 6·4 | | | 52·35 | 6·2 | | |
| 77 | $NO_2$ | S | 0 | $Pr^i$ | H | $Pr^i$ | H | 183·5 | | $C_{14}H_{20}N_8O_2S$ | 46·1 | 5·7 | 30·9 | 8·9 | 46·1 | 5·5 | 30·8 | 8·8 |
| 78 | $NO_2$ | NH | 0 | Et | H | $Pr^i$ | H | *b* 182 | (dec.) | $C_{13}H_{19}N_9O_2 \cdot \frac{1}{2}H_2SO_4 \cdot \frac{1}{2}H_2O$ | 40·3 | 5·5 | 32·4 | 4·0 | 39·9 | 5·4 | 32·2 | 4·1 |
| 79 | $NO_2$ | NH | 0 | $Pr^i$ | H | $Pr^i$ | H | 148–9 | | $C_{14}H_{21}N_9O_2 \cdot \frac{1}{4}H_2O$ | 48·1 | 6·2 | 36·0 | | 47·9 | 6·2 | 35·8 | |

*b* sulphate. *e* hydrochloride.

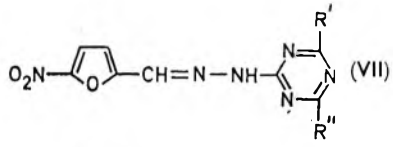



Table 4. *Di-substituted triazinylhydrazones of 5-nitro-2-furaldehyde*

| Cpd no. | R′ | R″ | m.p. | °C. | Formula | Found C | Found H | Found Cl | Found N | Required C | Required H | Required Cl | Required N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 80 | Cl | Cl | | | | | | | | | | | |
| 81 | OH | OH | | | | | | | | | | | |
| 82 | MeO | MeO | | | | | | | | | | | |
| 83 | Cl | NH·$Pr^i$ | 131–5 | | $C_{11}H_{12}ClN_7O_3$ | 40·6 | 3·9 | 10·7 | 30·1 | 40·6 | 3·7 | 10·9 | 30·1 |
| 84 | Cl | $NEt_2$ | | | | | | | | | | | |
| 85 | OH | $NHPr^i$ | *e* 253–5 | (dec.) | $C_{11}H_{18}N_7O_4 \cdot HCl \cdot 1{\cdot}6H_2O$ | 35·3 | 4·55 | | 26·4 | 35·6 | 4·7 | | 26·3 |
| 86 | OH | $NEt_2$ | | | | | | | | | | | |
| 87 | 2,4-Xylyl | 2,4-Xylyl | 189–190 | | $C_{24}H_{22}N_8O_3$ | 65·0 | 4·95 | | 19·1 | 65·1 | 5·1 | | 19·0 |
| 88 | Me | $NH_2$ | 240 | (dec.) | $C_9H_9N_7O_3 \cdot \frac{1}{4}H_2O$ | 40·3 | 3·7 | | 36·5 | 40·4 | 3·6 | | 36·6 |

*e* hydrochloride.

washed with water and dried. The dried solid was extracted with chloroform in a Soxhlet apparatus and the chloroform extract evaporated to low bulk. The crystals (2·9 g) which separated were collected by filtration and recrystallized from methanol (100 ml) to give yellow needles of the hydrazone (1·4 g), m.p. 215–6° (dec.) (Found: C, 42·6; H, 4·2; Cl, 10·6; N, 28·6. $C_{12}H_{14}ClN_7O_3$ Requires: C, 42·4; H, 4·15; Cl, 10·4; N, 28·9%).

2-*Diethylamino*-4-*hydroxy*-6-(5-*nitrofurfurylidenehydrazino*)-1,3,5-*triazine*. 2-Chloro-4-diethylamino-6-(5-nitrofurfurylidenehydrazino)-1,3,5-triazine (10 g) was dissolved in glacial acetic acid (100 ml) and the solution refluxed for 1½ h. The cooled solution deposited the hydrochloride of the product which was collected by filtration and washed with chloroform. The hydrochloride was dissolved in 50/50 aqueous methanol (150 ml) and neutralized with 2N sodium bicarbonate solution (70 ml). The precipitated base was collected by filtration and washed with water. The dried product was recrystallized from methanol (1200 ml) to give the hydrazone (3·5 g) as yellow plates, m.p. 257–263° (Found: C, 44·7; H, 4·8; N, 30·5. $C_{12}H_{15}N_7O_4$ Requires: C, 44·85; H, 4·7; N, 30·5%).

## BIOLOGICAL METHODS

### In vitro *assay for antituberculosis activity*

The compounds were dissolved in a suitable organic solvent at different concentrations. An exact amount of each solution was added to a hundredfold quantity of Youmans medium (Youmans & Karlson, 1947) containing 1·5% Noble—Agar (Difco) and 10% bovine serum. After thorough mixing, the compound-containing medium was distributed in culture-tubes (5 ml per tube) and slanted. After solidification the tubes were inoculated with standardized suspensions of tubercle bacilli using a wild strain ($A_5$) as well as an isoniazid-resistant ($INH_r$) and a streptomycin-resistant ($Str_r$) strain. The culture tubes were then incubated at 37° and read after 3 weeks. The minimal inhibitory concentration was determined which is the lowest concentration at which no growth occurred. Controls containing the same amount of solvent were included in each experiment.

### In vivo *assay of antituberculosis activity*

Albino mice, 20 g, of a strain susceptible to tuberculosis were infected intravenously with 0·2 ml of a suspension containing 0·8 mg virulent tubercle bacilli (wild strain $A_5$). Treatment was started 3 days after the infection and continued till the 33rd day after the infection. The compounds were suspended in 0·5% carboxymethylcellulose and given by stomach tube in dosages of 400, 200, 100 and 50 mg/kg during 5 days of the week. Groups of 10 mice were used for each dose-level. The life span after infection was noted and the median-survival time of each group compared with that of the untreated controls. A therapeutic effect was demonstrated by a significant increase of the survival time of the treated animals.

## RESULTS AND DISCUSSION

Of the compounds described in this paper all those which exhibited *in vitro* activity at a concentration of less than 10 μg/ml against at least one of the strains mentioned in the previous section are listed in Table 5.

Table 5. *Antituberculosis activity of nitrofuran derivatives*

| | *Mycobacterium tuberculosis* Minimum inhibitory concentration after 21 days (μg/ml) | | | |
|---|---|---|---|---|
| Cpd no. | $A_5$ | $INH_r$ | $Str_r$ | Activity *in vivo* $A_5$ |
| 3 | 0·5 | 10 | 2·5 | — |
| 4 | 0·3 | 3 | 0·3 | ++ |
| 5 | 1 | 10 | 1 | — |
| 6 | 0·25 | 2·5 | 0·25 | ++ |
| 7 | 0·25 | 2·5 | 0·5 | + |
| 8 | 0·1 | 1 | 0·25 | + |
| 9 | 0·25 | 2·5 | 0·5 | — |
| 10 | 1 | 10 | 1 | — |
| 11 | 1 | 2·5 | 0·5 | — |
| 12 | 0·25 | 1 | 0·25 | — |
| 13 | 0·1 | 1 | 0·1 | — |
| 14 | 0·25 | 0·5 | 0·25 | — |
| 15 | 1 | 2·5 | 2·5 | — |
| 16 | 0·25 | 1 | 0·25 | ++ |
| 17 | 0·3 | 1 | 0·3 | ++ |
| 18 | 0·25 | 1 | 0·25 | +++ |
| 19 | 0·25 | 2·5 | 0·25 | + |
| 20 | 1 | 5 | 1 | — |
| 21 | 0·3 | 3 | 1 | — |
| 22 | 0·25 | 1 | 0·25 | — |
| 23 | 0·25 | 2·5 | 0·5 | — |
| 29 | 1 | 10 | 1 | — |
| 30 | 1 | 10 | 10 | — |
| 31 | 1 | 10 | 10 | — |
| 32 | 0·1 | 1 | 0·1 | ++ |
| 33 | 0·5 | 2·5 | 2·5 | — |
| 34 | 1 | 10 | 2·5 | — |
| 36 | 0·25 | 0·5 | 1 | — |
| 37 | 2·5 | 5 | 5 | — |
| 38 | 0·25 | 2·5 | 0·25 | ++ |
| 39 | 5 | 10 | 5 | N.T. |
| 40 | 1 | 10 | 1 | — |
| 42 | 0·5 | >5 | 1 | — |
| 43 | 1 | 10 | 10 | — |
| 45 | 1 | 10 | 1 | — |
| 46 | 1 | 10 | 5 | — |
| 47 | 1 | 10 | 10 | — |
| 49 | 0·25 | 2·5 | 0·5 | — |
| 50 | 0·25 | 2·5 | 1 | + |
| 52 | 0·1 | 1 | 0·1 | — |
| 53 | 0·5 | 2·5 | 1 | — |
| 54 | 1 | 10 | 2·5 | — |
| 55 | 1 | 10 | 1 | — |
| 56 | 1 | 10 | 10 | N.T. |
| 59 | 0·03 | 0·3 | 0·03 | + |
| 60 | 0·03 | 0·1 | 0·03 | + |
| 62 | 0·3 | 0·3 | 0·3 | — |
| 63 | 0·3 | <1 | 0·3 | — |
| 64 | 3 | 10 | 3 | — |
| 65 | 1 | 3 | 3 | — |
| 70 | 1 | 3 | 0·3 | — |
| 72 | 1 | 1 | 1 | — |
| 73 | 3 | 3 | 3 | — |
| 77 | 0·5 | 1 | 0·5 | — |
| 83 | 0·1 | 2·5 | 0·25 | — |
| Isoniazid | 0·05–0·1 | >100 | 0·05–0·1 | ++++ |

The following criteria have been used in indicating the relative *in vivo* activities.

— = no activity observed up to a dose of 400 mg/kg per day. + = significant increase in survival-time of mice over controls at dose levels of 200–400 mg/kg. ++ = significant increase in survival-time of mice over controls at dose levels of 100 mg/kg per day and higher. +++ = significant increase in survival-time of mice over controls at dose levels of 50 mg/kg per day and higher. ++++ = significant increase in survival-time for mice over controls at a dose of 5 mg/kg per day. N.T. = not tested.

*Chemical structure—biological activity relations*

(a) *Diaminotriazinylhydrazones III, IV, and V.* The *in vitro* tests show a distinct relation between the antituberculosis activity and the structure of the diaminotriazinylhydrazones studied. In the series of amino(monosubstituted amino)triazinylhydrazones of Type III (cpds 1–15, Table 1) activity is quickly reached with the ethyl derivative (cpd 3) and only begins to fall with the 1-ethylpropyl derivative (cpd 10). The aryl derivatives (cpds 12–15) exhibit activity of a similar order to that of the $C_5$ alkyl derivative (cpd 8). A similar rise and fall of activity with increasing chain-length is noticed with the series of di(mono substituted amino)triazinylhydrazones of Type III (cpds 16–20, 22, 26, and 29–48, but particularly in the series formed by cpds 26, 22, 18, 42, 43, 45 and 44). The beneficial effect on activity of a secondary or tertiary alkyl group in this series is clearly illustrated by a comparison of cpds 16 with 30 and of 31 with 32 and 33; and many of the most active compounds synthesized contain an α-branched-chain component. The variation of one (substituted amino)-group when the other is the isopropylamino-group leads to no significant alteration in activity (cpds 16–19).

Amino (di-substituted amino)triazinylhydrazones of Type III are comparable with the isomeric di(monosubstituted amino)-compounds (cf. cpds 21 and 25 with 22 and 26).

The (monosubstituted amino) (disubstituted amino)triazinylhydrazones of Type III (except the trimethyl derivative, cpd 27) retain high activity (cpds 23, 52, and 53), but substitution of all four hydrogen atoms in the series of di(disubstituted amino) triazinylhydrazones leads to a weakening of activity (cpds 24, and 54–7). Relatively few compounds of Type III with high *in vitro* activity have *in vivo* activity. *In vivo* activity is essentially restricted to those compounds which possess at least one (α-branched-chain alkyl)amino-group or cycloalkylamino-group, and either two or three unsubstituted hydrogen atoms on the two amino-groups (cpds 4, 6–8, 16–19, 32 and 38).

The introduction of a methyl substitutent on the hydrazine nitrogen atom in compounds of Type IV (Table 2) led to an almost ten-fold increase in *in vitro* activity, closely approaching the activity of isoniazid. *In vivo* however the activity was reduced (cpds 59, 60 and 62). On the other hand introduction of a hydroxyethyl substituent in this position produced little effect (cpds 69 and 70). Two vinylogues (cpds 72 and 73) and a thiophene analogue (cpd 77) showed little difference in *in vitro* activity but *in vivo* activity was negligible. Furan and nitropyrrole analogues were without significant activity (cpds 74–6, and 78–9).

(b) *Other triazinylhydrazones type VII.* Replacement of the amino-group of a diaminotriazinylhydrazone by a halogen atom (as in cpd 83, Table 4; cf. cpd 4) maintained *in vitro* activity but not *in vivo* activity; but replacement of one amino-group by a hydroxyl group (as in cpd 85) resulted in loss of activity. Replacement of both amino-groups also caused loss of activity.

The structural requirements for antituberculosis activity are clearly quite critical. The nitro-group is essential, as is one amino-group or (substituted amino)-group on the triazine ring; for *in vivo* activity two amino-groups or (substituted amino)-groups are necessary.

REFERENCES

CIBA Ltd. (1961). British Patent 884, 802.

DODD, M. C., CRAMER, D. L., & WARD, W. C. (1950). *J. Am. pharm. Ass., Sci. Edn.*, **39**, 313–318.

ELLIS, G. P. & WEST, G. B. (1967). *Progress in Medicinal Chemistry*, Vol. 5, London: Butterworths.

HIRAO, I. & KATO, Y. (1964). *J. chem. Soc., Japan*, **85**, 231–235.

ICI Ltd. (1959). British Patent 816,886.

KREUTZBERGER, A. (1957). *J. Am. chem. Soc.*, **79**, 2629–2632.

PAUL, H. E. & PAUL, M. F. (1964). *Experimental Chemotherapy*, Vol. II, Part 1, p. 308, Editors, Schnitzer, R. J. & Hawking, F. London: Academic Press.

PEARLMAN, W. M. & BANKS, C. K. (1948). *J. Am. chem. Soc.*, **70**, 3726–3728.

SCHNITZER, R. J. (1964). *Experimental Chemotherapy*, Vol. II, Part I, p. 371–499, Editors, Schnitzer, R, J., & Hawking, F. London: Academic Press.

SENIER, A. (1886). *Ber.*, **19**, 310.

THURSTON, J. T., DUDLEY, J. R. KAISER, D. W., HECHENBLECKNER, I., SCHAFFER, F. C. & HOLM-HANSEN, D. (1951). *J. Am. chem. Soc.*, **73**, 2981–2983.

YOUMANS, G. P. & KARLSON, A. G. (1947). *Am. Rev. Tuberc. pulm. Dis.*, **55**, 529–535.

*J. Pharm. Pharmac.*, 1969, **21**, 434–440 *Received January* 13, 1969

# Structure-activity relations in analgesics based on 4-anilinopiperidine

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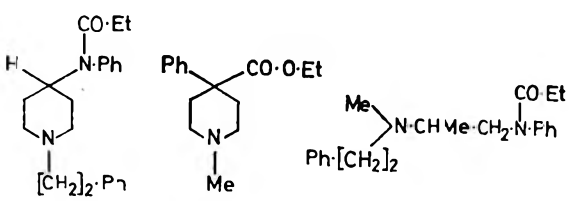
The synthesis of some 1-alkyl and 1-aralkyl-4-(*N*-phenylpropionamido)piperidines and related derivatives is described and the hot-plate activities in mice of these compounds reported. Activity variations among 1-methyl, 1-benzyl, and 1-phenethyl derivatives resemble those of corresponding open-chain anilide rather than 4-phenyl-piperidine analgesics. Infrared and nmr data show the two nitrogen atoms of the 4-anilinopiperidine derivatives to be further apart than those of open-chain anilides in their respective preferred conformations; these results, together with the extreme potency difference between the *N*-phenethyl derivatives diampromide and fentanyl, show that the two classes are best regarded as mutually distinct types of analgesic.

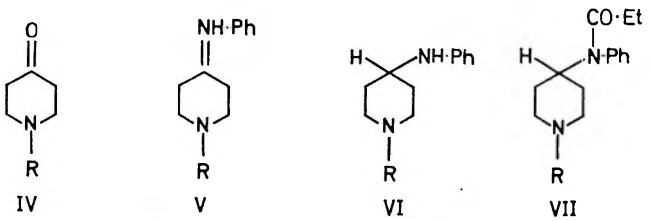
The analgesic fentanyl (I), introduced early this decade (Janssen, 1962), has proved a useful agent for the relief of pain and for neurolept analgesia, an anaesthetic technique in which an analgesic-tranquillizer mixture is given intravenously usually as an adjunct to nitrous oxide (Gorodetzky & Martin, 1965). It shows characteristic morphine-like effects e.g. Straub tail, mydriasis, and constipation in mice, and respiratory depression in dogs and cats, and appears to be a typical narcotic analgesic (Gardocki & Yelnosky, 1964; Gardocki, Yelnoksy & others, 1964). Fentanyl possesses molecular features of both 4-phenylpiperidine analgesics (e.g. pethidine, II) and open-chain basic anilides (e.g. diampromide, III, and its 2-thienyl analogue), and the present structure-activity study of 1-substituted 4-(*N*-phenylpropionamido)piperidines was made to obtain data which may help to establish more clearly its structural classification. This type of correlation among compounds with analgesic properties is of value, as it may lead to a better understanding of drug-receptor uptake modes and characteristics of the analgesic receptor (Portoghese, 1965; Casy, Simmonds & Staniforth, 1968).




I II III

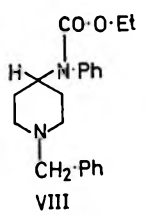
## CHEMISTRY

The 1 substituted 4-(*N*-phenylpropionamido)piperidines VII were obtained from 1-alkyl-or 1-aralkyl-4-piperidones IV by the three stage process IV to VII based upon a patent procedure (Janssen, 1964). Toluene-*p*-sulphonic acid was used as catalyst for




R= a) Me, b) $CH_2$·Ph, c) $[CH_2]_2$·Ph, d) $CH_2$·CH:$CH_2$,
e) $NMe_2$, f) $CH_2$CH:$CMe_2$, g) H

most piperidone-aniline condensations, but a better yield of the Schiff base Vb followed the use of zinc chloride, recently reported as a good catalyst for the synthesis of cyclohexane analogues (Bull, Hey & others, 1967). The Schiff bases V, characterized by strong $\nu_{CN}$ absorption bands at 1660 $cm^{-1}$, were acid-labile, the precursor piperidone (isolated as the diethyl acetal salt) being regenerated after attempts to make the hydrochloride of the Schiff base Vc. 4-(*N*-Phenylpropionamido)piperidine VIIg, obtained by catalytic debenzylation of the anilide VIIb, was also sensitive to acid, as shown by the loss of its acyl group after treatment with a slight excess of ethanolic hydrogen bromide. The ester VIII was prepared by reaction between 4-anilino-1-benzylpiperidine VIb and ethyl chloroformate, while the 1-allyl derivatives VIId and f were obtained by alkenylating 4-(*N*-phenylpropionamido)piperidine VIIg with the appropriate alkenyl halide.



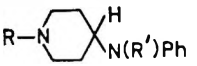

VIII

## PHARMACOLOGY AND DISCUSSION

The analgesic properties of the 4-acylaminopiperidines VII and related compounds were assessed in mice (Jacobson & May, 1965) by the hot-plate test (Table 1); thanks are due to Dr. E. L. May, National Institutes of Health, Bethesda, Maryland, for arranging these tests. Structure-activity relations are compared below with those of 4-phenylpiperidine analgesics and open-chain basic anilide analgesics.

Influences of the 1-substituent upon activity within compounds 1 (methyl), 3 (benzyl) and 5 (phenethyl) (Table 1) are not typical of 4-phenylpiperidine analgesics, as in these, 1-methyl derivatives commonly have significant potencies while 1-benzyl analogues are, at the most, only feebly active (Beckett & Casy, 1965). It is true that replacement of a 1-methyl group by a 1-phenethyl group enhances activity in compounds of the same class but the increases, typically 3–4 fold (Janssen & Eddy, 1960), are not nearly so great as that seen in the 4-anilinopiperidines (Table 1). Activity variations amongst compounds 1, 3 and 5 more closely follow those of corresponding open-chain basic anilides, in which aralkyl-amino-substituents are essential for high activity, and dimethylamino-derivatives have only feeble analgesic properties (Table 2). However,

Table 1. *Analgesic activities of some 4-anilinopiperidines measured by the hot-plate test in mice after subcutaneous injection**



| No. | R | R′ | ED50 mg/kg |
|---|---|---|---|
| 1 | Me | CO·Et | Inactive (100 mg/kg) |
| 2 | Me | H | 7·5 |
| 3 | $CH_2$·Ph | CO·Et | 10·45 |
| 4 | $CH_2$·Ph | CO·O·Et | 78·0 |
| 5 (Fentanyl) | $[CH_2]_2$·Ph | CO·Et | 0·01 |
| 6 | $CH_2$·CH:$CH_2$ | CO·Et | 12·1 |
| 7 | $CH_2$·CH:$CMe_2$ | CO·Et | 9·8 |
| 8 | $NMe_2$ | CO·Et | inactive (100 mg/kg) |

* Method of Jacobson & May (1965); *cf.* pethidine ED50 4·7 mg/kg

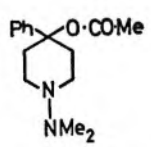
Table 2. *Analgesic activities of some open-chain basic anilides*

MeN·CHMe·$CH_2$·NAr
R R′

| | | | Activity (mg/kg) | |
|---|---|---|---|---|
| Ar | R | R′ | AD50 (tail flick)* | ED50 (hot plate)†‡ |
| Ph | Me | CO·Et | — | 50 |
| Ph | $CH_2$·Ph | CO·Et | 8 | 15 |
| Ph | $[CH_2]_2$·Ph | CO·Et | 3·7 | — |
| 2-Thienyl | Me | CO·Et | — | 46·5 |
| 2-Thienyl | $CH_2$·Ph | CO·Et | — | 29·9 |
| 2-Thienyl | $[CH_2]_2$·Ph§ | CO·Et | — | 7·1 |
| 2-Thienyl | $[CH_2]_2$·Ph | CO·O·Et | — | >100 |

* In rats (Wright and Hardy, 1963); pethidine AD50 = 11 mg/kg
† in mice (Casy and Hassan, 1967); pethidine ED50 = 23 mg/kg
‡ Thienyl derivatives: in mice (Sugimoto & others, 1962); morphine hydrochloride ED50 = 6·8 mg/kg
§ MeNR·CHMe·$[CH_2]_2$·NR′Ar analogue, ED50 57·7 mg/kg

although replacement of a 1-benzyl group by a 1-phenethyl group enhances activity in both the 4-anilinopiperidines and the open-chain basic anilides, the degree of potency rise is far greater in the 4-anilinopiperidines (the potency rise is 2–4 fold in the open-chain basic anilides but over 800 in the 4-anilinopiperidines). The dimethylamino-piperidine derivative IX related to a reversed ester of pethidine is about two-thirds as active as pethidine in the mouse hot-plate test (Beckett & Greenhill, 1961), a result in contrast with the complete inactivity of the 4-anilinopiperidine compound 8 (Table 1). The large decrease in activity following replacement of the *N*-propionyl group in compound 3 by the ethoxycarbonyl group (compound 4) is also seen in open-chain basic anilides (Table 2).



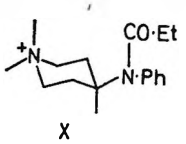
IX

The significant activity of the 1-allyl and the 1-(3-methylbut-2-enyl) derivatives, compounds 6 and 7 (Table 1), distinguishes the 4-anilinopiperidine analgesics from the morphine, morphinan and benzomorphan class, *N*-allyl derivatives of which are analgesic antagonists rather than agonists in animals. The analgesic potencies of compounds 6 and 7 are, however, considerably lower than those of comparable 4-phenylpiperidines (Casy & others, 1968).

The relative potencies of compounds 1 and 2 are unusual in that the compound lacking an *N*-propionyl function [established as the optional substituent in open-chain basic anilide analgesics (Sugimoto, Okumura & others, 1962)] is only slightly less active than pethidine, while its *N*-acylated analogue is completely inactive. Further data upon the analgesic properties of related diamines are being sought.

It is concluded from these pharmacological results that heterocyclic-substituted anilide analgesics based on 4-anilinopiperidine are more closely related to open-chain basic anilide analgesics than to 4-phenylpiperidine analgesics; the two classes of anilide differ, however, in the large potency discrepancy between the heterocyclic and open chain 1-phenethyl derivatives.

Further evidence bearing upon the common classification of open-chain basic and heterocyclic-substituted anilide analgesics may be obtained by a comparison of their preferred conformations. If the relative orientations of key functions in the two classes prove to be alike, support is provided for their having similar uptake modes at the receptor. Although agonists do not necessarily associate with their receptors in the more stable conformations (binding forces offsetting conformational energy barriers), it seems reasonable, as an initial approach, to compare conformers that are likely to be the more highly populated under physiological conditions. On steric grounds more favoured conformers of 4-anilinopiperidine derivatives are predicted to be piperidine-chairs with equatorial anilido-groups (X), and support for this conclusion

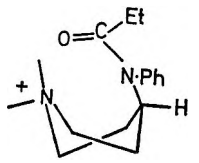



X

is provided from the following spectroscopic evidence. In the nmr spectra of bases and salts of the derivatives VIIa, VIIb and VIIc, 4-methine proton signals near 5$\tau$ are resolvable because they are moved downfield from ring proton signals, etc. by the adjacent deshielding anilide substituent. All the methine signals are broad multiplets of about 36 Hz width, clear nonets being obtained for the 1-methyl and 1-phenethyl bases from which coupling constants near 4–5 and 11·0 Hz may be abstracted (assuming an approximately first order system). Signal widths and coupling constants of these orders are consistent with the operation of 2 axial-axial and 2 axial-equatorial spin-spin coupling interactions regarding the 4-methine signal (Bhacca & Williams, 1964; Chen & Le Fèvre, 1965) and support X as the preferred conformation of 4-anilinopiperidine derivatives. This conclusion refers to bases and hydrochloride salts as solutes in $CDCl_3$; data in water would be of greater pharmacological significance but unfortunately the methine signals of hydrochloride salts in $D_2O$ were obscured by the residual water signal.

In open-chain basic anilides (e.g. III), evidence for the proximity of the two nitrogen atoms was provided by the significantly greater $\nu_{C=O}$ stretching frequency values of hydrochloride salts compared with those of free bases (Casy & Hassan, 1967). In

contrast, the hydrochloride and base of the heterocyclic-substituted anilide VIIb had almost identical $\nu_{C=O}$ values as solutes in $CHCl_3$ (see experimental) and this result shows that the population of flexible conformers of type XI (where the protonated nitrogen atom may be close enough to the anilido-nitrogen atom to influence the $\nu_{C=O}$ value) must be low.




XI

As noted above, there is evidence that the two nitrogen atoms of open-chain basic anilides (separated by a two-carbon chain) are close together in their preferred conformations* and the *N*-to-*N* distance may well have a significant influence upon activity in the open-chain class as analogues in which the nitrogen atoms are separated by a three-carbon chain are only feebly active (Table 2). A carbon chain of the same length connects the nitrogen atoms of fentanyl and its congeners but these centres may approach each other if the molecule adopts the skew-boat conformation XI. Spectroscopic evidence already given, however, clearly supports the chair form X and provides no support for flexible conformers.

Conformational considerations do not, therefore, uphold the like classification of open-chain basic and heterocyclic-substituted anilide analgesics and this evidence, together with the extreme potency difference between the *N*-phenethyl derivatives diampromide and fentanyl, show that the two classes are best regarded as mutually distinct types of analgesic.

## EXPERIMENTAL

Salts crystallized from acetone or ethanol-ether unless otherwise stated.

*1-Benzyl-4-(N-phenylpropionamido)piperidine and related compounds.* A mixture of 1-benzyl-4-piperidone IVb (28·5 g), aniline (18 g), toluene-*p*-sulphonic acid (15 mg) and toluene (240 ml) was heated under reflux for 15 h and then fractionally distilled to give the Schiff base Vb (10·5 g, 37%), b.p. 210°/0·5 mm (Janssen, 1964, gives b.p. 170°/0·05 mm) (Found: C, 81·5; H, 7·8; N, 10·4. $C_{18}H_{20}N_2$ requires: C, 81·8; H, 7·6; N, 10·6%). A mixture of IVb (57 g), aniline (36 g), zinc chloride (0·1 g) and xylene (500 ml) gave the Schiff base Vb in 53% yield (42 g) after a 24 h reflux period. A mixture of Vb (31·2 g), lithium aluminium hydride (9·6 g) and ether (500 ml) was heated under reflux for 5 h, then cooled, decomposed with water and filtered. The product, 4-anilino-1-benzylpiperidine VIb (35 g), m.p. 85–86° (Janssen, 1964, gives m.p. 84·8–86°) gave a *dihydrochloride*, m.p. 318–319° (Found: C, 64·3; H, 7·3. $C_{18}H_{24}Cl_2N_2$ requires C, 63·7; H, 7·1%). A mixture of VIb (15 g), propionic anhydride (10 g) and benzene (200 ml) gave 1-benzyl-4-(*N*-phenylpropionamido)piperidine VIIb (18 g) after a 3 h reflux period. It formed a *hydrochloride*, m.p. 235–237° (Found: C, 70·7; H, 7·6; N, 7·6. $C_{21}H_{27}ClN_2O$ requires: C, 70·3, H, 7·6; N, 7·8%). The following related compounds were prepared similarly:

* The physical possibility of the two nitrogen atoms being in juxtaposition has been demonstrated by an X-ray analysis of (+)-*N*-[2-(benzylmethylamino)propyl]propionanilide hydrobromide, in which the *N*–*N* distance (3·02 Å) barely exceeds the sum of the two van der Waals' radii (3·0 Å) (Ahmed, personal communication).

1-*Methyl*-4-(N-*phenylpropionamido*)*piperidine* VIIa, b.p. 126–128°/0·1 mm (Found: C, 73·6; H, 9·2; N, 11·6. $C_{15}H_{22}N_2O$ requires: C, 73·1; H, 9·0; N, 11·4%), *hydrochloride*, m.p. 265–266° (Found: C, 62·0; H, 8·5; N, 10·3. $C_{15}H_{23}ClN_2O$ requires: C, 62·1; H, 8·6; N, 10·35%) from the *Schiff base* Va, b.p. 134–136°/0·5 mm (Found: C, 76·5; H, 9·0; N, 15·0. $C_{12}H_{16}N_2$ requires: C, 76·5; H, 8·6; N, 14·9%) and 4-*anilino*-1-*methylpiperidine* VIa, m.p. 81–82° from aqueous ethanol (Found: C, 75·5, H, 9·5; N, 14·9. $C_{12}H_{18}N_2$ requires: C, 75·7; H, 9·5; N, 14·7%), *dihydrochloride*, m.p. 260–262° (Found: C, 54·3; H, 7·8; N, 10·35. $C_{12}H_{20}Cl_2N_2$ requires: C, 54·75; H, 7·7; N, 10·6%).

1-*Phenethyl*-4-(N-*phenylproprionamido*)*piperidine* VIIc, m.p. 82–84° (Janssen, 1964, gives m.p. 83–84°), *hydrochloride*, m.p. 254–255 (Found: C, 71·0; H, 7·75; N, 7·3. $C_{22}H_{29}ClN_2O$ requires: C, 70·9; H, 7·8; N, 7·5%) from the Schiff base Vc, b.p. 198–200°/0·4 mm and 4-*anilino*-1-*phenethylpiperidine* VIc, m.p. 90–91° from light petroleum (b.p. 60–80°) (Found: C, 80·9; H, 8·65; N, 9·95. $C_{19}H_{24}N_2$ requires: C, 81·4; H, 8·6; N, 10·0%), *dihydrochloride*, m.p. 261–262° from methanol (Found: C, 64·4; H, 7·4; N, 7·9. $C_{19}H_{26}Cl_2N_2$ requires: C, 64·6; H, 7·4; N, 7·9%).

The 1-*Dimethylamino*-4-(N-*phenylpropionamido*)*piperidine* VIIe, b.p. 183°/1 mm (Found: C, 69·4; H, 9·1; N, 15·3. $C_{16}H_{25}N_3O$ requires: C, 69·8; H, 9·1; N, 15·3%), *hydrobromide*, m.p. 193° (Found: C, 53·9; H, 7·1; N, 11·9. $C_{16}H_{26}BrN_3O$ requires: C, 53·9; H, 7·3; N, 11·8%) from the *Schiff base* Ve, b.p. 180°/1·3 mm (Found: C, 71·2; H, 9·1. $C_{13}H_{19}N_3$ requires: C, 71·8; H, 8·8%) and 4-*anilino*-1-*dimethylaminopiperidine* VIe, b.p. 189°/2 mm (Found: C, 70·6; H, 9·4. $C_{13}H_{21}N_3$ requires C, 71·2; H, 9·6; *hydrobromide*, m.p. 220° (Found: C, 52·1; H, 7·4; N, 13·8. $C_{13}H_{22}BrN_3$ requires: C, 52·0; H, 7·4; N, 14·0%).

The Schiff base Vc was converted to the diethyl acetal hydrochloride of 1-phenethyl-4-piperidone IVc, m.p. 184–186° (Beckett & others, 1959, give m.p. 178–179°) (Found: C, 64·7; H, 8·9; N, 4·5. Calc. for $C_{17}H_{28}ClNO_2$: C, 65·1; H, 9·0; N, 4·5%) on treatment with ethanolic hydrogen chloride.

1-*Alkylations of* 4-N-*phenylpropionamidopiperidine*. A mixture of 1-benzyl-4-(*N*-phenylpropionamido)piperidine VIIb (13 g), palladized charcoal (2 g, 5%) and ethanol (200 ml) was shaken with hydrogen (room temperature, atmospheric pressure) until gas absorption ceased. The suspension was filtered and the filtrate evaporated to give 4-(*N*-phenylpropionamido)piperidine VIIg (7 g), m.p. 84–85° (Janssen, 1964, gives m.p. 83–85°). It was converted to 4-*anilinopiperidine* (VIg) *dihydrobromide*, m.p. 266–268° (Found: C, 39·9; H, 5·65; N, 8·9. $C_{11}H_{18}Br_2N_2$ requires: C, 39·1; H, 5·4; N, 8·3%) on treatment with ethanolic hydrogen bromide. A mixture of VIIg (7 g), allyl bromide (4 g), sodium bicarbonate (13 g) and acetone (200 ml) was stirred and heated under reflux for 11 h. The cooled mixture was filtered, the filtrate evaporated and the residue acidified with hydrogen bromide in isopropanol to give 1-*allyl*-4-(N-*phenylpropionamido*)*piperidine* (VIId) *hydrobromide*, m.p. 199° (Found: C, 57·8; H, 6·9; N, 7·6. $C_{17}H_{25}BrN_2O$ requires: C, 57·8; H, 7·1; N, 7·9%). A similar alkylation of VIIg with 1-chloro-3-methylbut-2-ene gave 1-(3-*methylbut*-2-*enyl*)-4-(N-*phenylpropionamido*)*piperidine* (VIIf) *hydrobromide*, m.p. 233 (Found: C, 59·6; H, 7·2; N, 7·5. $C_{19}H_{29}BrN_2O$ requires: C, 59·8; H, 7·1; N, 7·4%).

1-*Benzyl*-4-(N-*ethoxycarbonyl*-N-*phenylamino*)*piperidine*. A mixture of 4-anilino-1-benzylpiperidine VIb (13·3 g), ethyl chloroformate (7·65 g) and benzene (50 ml) was heated under reflux for 3 h and then evaporated to dryness. The residue was made

alkaline with aqueous potassium hydroxide solution and extracted with ether. The *N*-ethoxycarbonyl derivative VIII (9·5 g), recovered from the extract, formed a *hydrochloride*, m.p. 229–230° (Found: C, 67·4; H, 7·2; N, 7·2. $C_{21}H_{27}ClNO_2$ requires: C, 67·3; H, 7·3; N, 7·5%).

*Infrared data upon 1-benzyl-4(N-phenylpropionamido) piperidine.*

*Base* $\nu_{C=O}$ 1650 $cm^{-1}$ (film), 1637 $cm^{-1}$ (10, 5, 3 and 1% solution in chloroform).

*Hydrochloride* $\nu_{C=O}$ 1654 $cm^{-1}$ (Nujol mull), 1636 ± 1 $cm^{-1}$ (10, 5, 3, 1, 0·5, 0·3 and 0·1% solution in chloroform).

Infrared spectra were recorded on a Unicam S.P. 100 spectrometer and nmr spectra on a Varian A-60 spectrophotometer using deuterochloroform as solvent and TMS as internal standard.

*Acknowledgement*

Two of us (A.F.C. and M.M.A.H.) thank the Canadian Foundation for the Advancement of Pharmacy for financial support.

## REFERENCES

BECKETT, A. H., CASY, A. F. & KIRK, G. (1959). *J. mednl pharm. Chem.*, **1**, 37–58.

BECKETT, A. H. & CASY, A. F. (1965). *Progress in Medicinal Chemistry*, and references cited. Volume 4, p. 171, Editors: Ellis G. P. & West, G. B. London: Butterworths.

BECKETT, A. H. & GREENHILL, J. (1961). *J. mednl pharm. Chem.*, **4**, 423–436.

BHACCA, N. S. & WILLIAMS, D. H. (1964). Applications of NMR spectroscopy in organic chemistry, San Francisco: Holden-Day.

BULL, J. R., HEY, D. G. MEAKINS, G. D. & RICHARDS, E. E. (1967). *J. chem. Soc.*, C, 2077–2081.

CASY, A. F., SIMMONDS, A. B. & STANIFORTH, D. (1968). *J. Pharm. Pharmac.*, **20**, 768–774.

CASY, A. F. & HASSAN, M. M. A. (1967). *Ibid.*, **19**, 17–24, 114–123.

CHEN, C.-Y. & LE FÈVRE, R. J. W. (1965). *J. chem. Soc.*, 3467–3473; 3473–3477.

GARDOCKI, J. K. & YELNOSKY, J. (1964). *Toxic. appl. Pharmac.*, **6**, 48–62.

GARDOCKI, J. K., YELNOSKY, J., KUEHN, W. F. & GUNSTER, J. C. (1964). *Ibid.*, **6**, 593–601.

GORODETZKY, C. W. & MARTIN, W. R. (1965). *Clin. Pharmac. Ther.*, **6**, 731–739.

JACOBSON, A. E. & MAY, E. L. (1965). *J. mednl Chem.*, **8**, 563–566.

JANSSEN, P. A. J. (1962). *Br. J. Anaesthesia*, **34**, 260–268.

JANSSEN, P. A. J. (1964). U.S. Patent 3, 141, 823, *Chem. Abstr.*, **62**, 14634c–14635b.

JANSSEN, P. A. J. & EDDY, N. B. (1960). *J. mednl pharm. Chem.*, **2**, 31–45.

PORTOGHESE, P. S. (1965). *J. mednl Chem.*, **8**, 609–616.

SUGIMOTO, N., OKUMURA, K., SHIGEMATSU, N. & HAYSHI, G. (1962). *Chem. Pharm. Bull., Tokyo*, **10**, 1061–1064.

WRIGHT, W. B. & HARDY, R. A. (1963). *J. mednl Chem.*, **6**, 128–130.

*J. Pharm. Pharmac.*, 1969, **21**, 441-450 *Received February* 10, 1969

# Methaemoglobin formation by aromatic amines

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Methaemoglobin formation induced in cats by many substituted anilines has been investigated in an attempt to correlate chemical structure with activity. Although no precise relation emerged, some generalizations could be made. 4-Substituents (except methyl and methoxy) increased the activity of aniline. The activity of aniline was either unaltered or reduced by 2- and 3-substituents. Polyhalo-, polymethyl-, methoxy-, ethoxy- and carboxyester substituents reduced activity, and carboxy groups abolished it. Steric effects around the amino-group were not important. The activities of the compounds are discussed in relation to their absorption, distribution, metabolic transformation and the activities of their metabolites.

Although many aromatic amines have been examined for their methaemoglobin-forming activity over the past 50 years (reviewed by Bodansky, 1951; Kiese, 1966), the results of different workers are not directly comparable since the animal species used and the experimental conditions varied. In an attempt to correlate chemical structure with methaemoglobin-forming activity, many ring substituted derivatives of aniline were administered to the cat and the methaemoglobin responses obtained compared with that observed for aniline given by the same route and at the same molar dose level.

## EXPERIMENTAL

### *Chemical*

Some amines were obtained commercially. These were purified and characterized by their physical constants and those of their derivatives. Other amines were prepared by standard methods and purified until acceptable elemental analyses were obtained (McLean, 1963). The acetanilide and isobutyranilide used were described previously (McLean, Murphy & others, 1967).

### *Biological*

Cats were used because of their sensitivity to methaemoglobin formation (Lester, 1943; Spicer, 1950). Only adult cats (older than 24 weeks) were used, since new-born kittens have been shown to reduce methaemoglobin faster than adult cats (Müller-Oerlinghausen & Baethke, 1966). Methaemoglobin determination, administration of compounds and general procedure was as described by McLean & others (1967).

## RESULTS

*Methaemoglobin in untreated cats.* Of the 152 animals examined, nearly all (92%) had 3% or less of their haemoglobin as methaemoglobin; the average value (1·1%) being less than the limit of sensitivity of the assay (1·3%).

*Irreversible destruction of haemoglobin.* After experiments in which methaemoglobin formation induced by a representative sample of aromatic amines and amides

was followed for several hours, the level of "intact" haemoglobin (oxy- plus methaemoglobin) (Robin & Harley, 1964) was, on average, 95% of the initial value (Table 1).

Table 1. *"Intact" haemoglobin (oxy-plus methaemoglobin) after methaemoglobin formation induced by various drugs expressed as a percentage of initial haemoglobin concentration*

| Compound | Dose mmol/kg | Route | No. cats | Mean % methaemoglobin formed | Duration of expt. (h) | Mean % "Intact" Hb |
|---|---|---|---|---|---|---|
| Acetanilide .. .. | 1·25 | oral | 1 | 60·1 | 8 | 70·7 |
| Acetanilide .. .. | 1·0 | oral | 1 | 66·1 | 6 | 111·6 |
| i-Butyranilide .. | 1·0 | oral | 1 | 78·0 | 6 | 117·8 |
| Acetanilide + .. | 1·0 | oral | 1 | 82·3 | 6 | 110·3 |
| SKF 525A* | 60 mg | i.p. | | | | |
| 2,4-Dimethylaniline .. | 0·25 | i.v. | 5 | 6·3 | 5 | 91·9 |
| 2,4-Difluoroaniline .. | 0·25 | i.v. | 3 | 62·5 | 5 | 95·2 |
| 4-Fluoroaniline .. | 0·25 | i.v. | 3 | 66·0 | 5 | 84·4 |
| 3-Bromoaniline .. | 0·25 | oral | 5 | 46·6 | 5 | 93·6 |
| Lignocaine .. .. | 0·20 | i.v. | 4 | 6·0 | 4 | 96·1 |
| Prilocaine .. .. | 0·20 | i.v. | 4 | 50·5 | 4 | 99·9 |

* Administered 45 min before acetanilide.

*Methaemoglobin formation by aniline*

*Route of administration.* Table 2 shows the formation of methaemoglobin after oral and intravenous administration of aniline at 0·25 and 0·0625 mmol/kg. A Student's *t*-test showed that the maximum methaemoglobin response after intravenous administration at 0·25 mmol/kg was higher than that after oral administration ($P < 0·02$). At 0·0625 mmol/kg, the difference in maximum response was not significant ($P > 0·5$), although the first hour mean was lower after oral administration ($P = 0·05$).

Table 2. *Formation of methaemoglobin after administration of aniline*

| Dose mmol/kg | No. cats. | Route admin. | Methaemoglobin % ± s.e. after time: 1 h | 2 h | 3 h | 4 h | 5 h | Mean | Mean max. |
|---|---|---|---|---|---|---|---|---|---|
| 0·25 | 9 | i.v. | 65·1 | 67·6 | 63·4 | 58·2 | 53·7 | 61·6 | 72·3 |
| | | | 5·1 | 1·3 | 3·6 | 3·0 | 2·8 | | 4·1 |
| 0·0625 | 19 | i.v. | 34·5 | 33·5 | 24·2 | 19·5 | 12·8 | 24·9 | 36·8 |
| | | | 1·6 | 2·3 | 2·5 | 2·6 | 2·3 | | 1·7 |
| 0·25 | 5 | Oral | 45·7 | 49·1 | 52·5 | 49·5 | 43·9 | 48·1 | 53·2 |
| | | | 4·1 | 3·8 | 4·0 | 3·2 | 2·7 | | 3·8 |
| 0·0625 | 5 | Oral | 25·4 | 30·6 | 28·0 | 24·8 | 17·5 | 25·2 | 33·9 |
| | | | 5·8 | 5·3 | 3·9 | 4·6 | 2·8 | | 5·0 |

*Time to maximum response.* To determine when the maximum response occurred, aniline (0·0625 mmol/kg) was given intravenously to 5 cats and the methaemoglobin concentration determined at 10 min intervals for 90 min, and thereafter at 120 and 150 min. The results obtained are shown in Fig. 1. The maximum methaemoglobin level was reached between the first and second hour in every animal and remained fairly constant during this period.

*Methaemoglobin formation by substituted anilines*

*Halogen substituted anilines.* The results obtained with the haloanilines are shown in Table 3 from which it can be seen that 3- and 4-fluoroaniline and 2,4-difluoroaniline formed as much methaemoglobin as aniline, while 2-fluoroaniline and 2,5-difluoroaniline were less active. Trifluoromethyl-, tetrafluoro- and pentafluoroaniline were

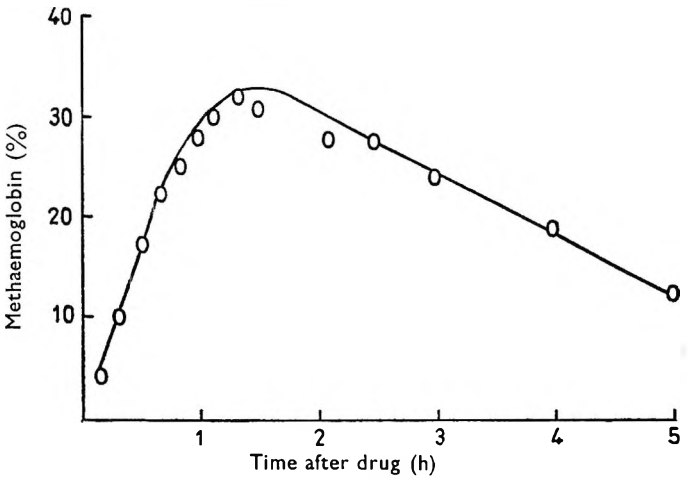


FIG. 1. The mean methaemoglobin percentage formed in cats after intravenous administration of aniline at 0·0625 mmol/kg. Each point is the mean of values from 5 cats.

Table 3. *Formation of methaemoglobin by halo-anilines*

| Compound | Dose mmol/kg | Route | No. cats | Methaemoglobin % ± s.e. after time : 1 h | 2 h | 3 h | 4 h | 5 h | Mean | Mean max. |
|---|---|---|---|---|---|---|---|---|---|---|
| 2-Fluoroaniline | 0·25 | i.v. | 5 | 44·2 | 51·3 | 48·0 | 46·4 | 37·5 | 45·5 | 52·9 |
| | | | | 2·5 | 4·3 | 3·0 | 4·3 | 6·1 | | 4·0 |
| 3-Fluoroaniline | 0·25 | i.v. | 5 | 46·5 | 65·2 | 65·0 | 63·4 | 63·7 | 60·8 | 69·1 |
| | | | | 4·3 | 1·9 | 4·0 | 4·3 | 4·4 | | 3·0 |
| 4-Fluoroaniline | 0·25 | i.v. | 5 | 64·3 | 73·2 | 66·1 | 63·0 | 63·4 | 66·0 | 74·9 |
| | | | | 3·3 | 4·7 | 5·9 | 5·9 | 3·4 | | 4·5 |
| 2,4-Difluoroaniline | 0·25 | i.v. | 5 | 69·6 | 61·7 | 62·5 | 62·2 | 56·6 | 62·5 | 71·4 |
| | | | | 6·5 | 4·3 | 2·4 | 6·3 | 5·1 | | 5·2 |
| 2,5-Difluoroaniline | 0·0625 | i.v. | 5 | 16·0 | 12·1 | 8·7 | 5·0 | 3·5 | 9·0 | 16·0 |
| | | | | 2·4 | 1·3 | 0·9 | 1·0 | 0·5 | | 2·4 |
| 2-Trifluoromethylaniline | 0·0625 | Oral | 3 | 5·1 | 3·1 | 1·5 | 0·9 | 0·5 | 2·2 | 5·5 |
| | | | | 2·3 | 0·8 | 0·2 | 0·1 | 0·2 | | 2·1 |
| 3-Trifluoromethylaniline | 0·0625 | Oral | 3 | 8·2 | 7·3 | 5·8 | 5·6 | 5·3 | 6·4 | 8·8 |
| | | | | | | | | | | 2·8 |
| Pentafluoroaniline | 0·25 | Oral | 1 | 0 | 1·1 | 3·3 | 1·8 | 0·7 | 1·4 | 3·3 |
| | 0·0625 | Oral | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2,3,4,5-Tetrafluoroaniline | 0·25 | Oral | 1 | 4·0 | 3·0 | 2·4 | 4·9 | 2·4 | 3·3 | 4·9 |
| | 0·0625 | Oral | 1 | 2·9 | 2·3 | 0 | 1·0 | 0·6 | 1·4 | 2·9 |
| 2,3,5,6-Tetrafluoroaniline | 0·25 | Oral | 1 | 9·0 | 8·6 | 10·7 | 13·4 | 10·3 | 10·4 | 13·4 |
| | 0·0625 | Oral | 1 | 1·3 | 1·6 | 0 | 0 | 0 | 0·6 | 1·6 |
| 2-Chloroaniline | 0·25 | Oral | 5 | 57·6 | 62·0 | 57·1 | 47·3 | 41·2 | 53·1 | 64·9 |
| | | | | 2·2 | 4·1 | 3·2 | 5·3 | 7·7 | | 2·7 |
| 3-Chloroaniline | 0·25 | Oral | 5 | 24·8 | 39·9 | 46·3 | 53·1 | 58·0 | 47·3 | 60·4* |
| | | | | 2·1 | 5·1 | 5·4 | 5·2 | 6·8 | | 6·2* |
| 4-Chloroaniline | 0·0625 | Oral | 5 | 17·1 | 33·1 | 46·4 | 53·8 | 57·8 | 45·2 | 60·7† |
| | | | | 3·1 | 6·0 | 7·5 | 7·6 | 8·1 | | 8·1† |
| 2,4-Dichloroaniline | 0·25 | Oral | 5 | 39·3 | 37·3 | 31·5 | 27·6 | 22·6 | 31·7 | 42·0 |
| | | | | 9·3 | 9·4 | 8·2 | 8·6 | 7·6 | | 9·9 |
| 2,6-Dichloroaniline | 0·25 | Oral | 5 | 14·3 | 22·2 | 20·4 | 18·7 | 13·5 | 17·0 | 23·3‡ |
| | | | | 2·1 | 5·4 | 4·7 | 6·1 | 5·0 | | 4·7‡ |
| 2,4,6-Trichloroaniline | 0·25 | Oral | 5 | 35·9 | 43·7 | 39·7 | 37·6 | 36·8 | 38·8 | 44·3 |
| | | | | 4·7 | 5·6 | 5·2 | 4·5 | 6·9 | | 5·5 |
| 2-Bromoaniline | 0·25 | Oral | 5 | 58·8 | 59·0 | 57·2 | 54·9 | 52·2 | 56·4 | 65·3 |
| | | | | 8·0 | 4·8 | 2·5 | 3·2 | 3·7 | | 4·7 |
| 3-Bromoaniline | 0·25 | Oral | 5 | 23·3 | 40·8 | 50·9 | 50·5 | 59·8 | 46·6 | 62·0§ |
| | | | | 4·2 | 7·7 | 8·5 | 8·0 | 7·5 | | 8·2§ |
| 4-Bromoaniline | 0·0625 | Oral | 5 | 14·6 | 29·7 | 43·8 | 49·7 | 52·6 | 42·2 | 56·1‖ |
| | | | | 1·8 | 2·8 | 4·6 | 6·2 | 5·9 | | 4·7‖ |
| 2,6-Dibromoaniline | 0·25 | Oral | 5 | 3·5 | 5·5 | 2·7 | 2·3 | 0·7 | 2·6 | 6·0¶ |
| | | | | 0·8 | 1·7 | 2·0 | 2·1 | 0·5 | | 1·6¶ |
| 2,4,6-Tribromoaniline | 0·25 | Oral | 5 | 18·2 | 21·7 | 17·7 | 20·7 | 17·5 | 19·1 | 23·6 |
| | | | | 5·5 | 5·8 | 6·3 | 7·9 | 5·6 | | 6·5 |

Some mean results include measurements made over 5 h, these are:
* 56·6 ± 4·5 at 6 h, 52·2 ± 4·3 at 7 h
† 54·5 ± 8·2 at 6 h, 51·4 ± 9·5 at 7 h, 47·8 ± 9·6 at 8 h.
‡ 12·6 ± 3·9 at 6 h.
§ 54·6 ± 7·0 at 6 h.
‖ 54·4 ± 5·3 at 6 h, 50·8 ± 3·8 at 7 h.
¶ 0·9 ± 0·9 at 6 h.

only weakly active. The time course of the methaemoglobin response to the fluoroanilines was similar to that found for aniline (Table 2). 3-Chloroaniline was as active as aniline, while 4-chloroaniline was much more active A Student's *t*-test showed that 2-chloroaniline produced a higher maximum level of methaemoglobin than aniline ($P < 0.05$). The mean maximum response to 2,4-dichloroaniline did not differ significantly from that to aniline ($P > 0.3$) or 2,6-dichloroaniline ($P > 0.1$), due partly to the high variance of the response to 2,4-dichloroaniline. The maximum methaemoglobin response to 2,4,6-trichloroaniline was not significantly different from that to 2,4-dichloroaniline and aniline ($P > 0.2$) but was greater than that to 2,6-dichloroaniline ($P = 0.02$). Aniline was more active than 2,6-dichloroaniline ($P < 0.01$).

The pattern of the responses to the various bromoanilines was similar to that observed for the corresponding chloroanilines; 2- and 3-bromoaniline were as active as aniline, and 4-bromoaniline was much more active. 2,4,6-Tribromoaniline formed less methaemoglobin than aniline and 2,6-dibromoaniline formed less than 2,4,6-tribromoaniline ($P < 0.05$). Although the monobromoanilines formed the same amount of methaemoglobin as the corresponding chloroanilines, 2,6-dibromoaniline and 2,4,6-tribromoaniline formed less methaemoglobin than the corresponding chloroanilines. All the chloro- and bromoanilines tended to produce a long-lasting methaemoglobin response, especially the 3- and 4-haloanilines which produced a maximum response in the fifth or sixth hour.

*Alkyl substituted anilines.* Methyl substituents tended, if anything, to lower the methaemoglobin forming activity of aniline (Table 4). Although the rate of formation of methaemoglobin after 3-methylaniline was slower than after aniline, 2- or 4-methylaniline, the log dose-maximum response curves for 3-methylaniline and aniline

Table 4. *Formation of methaemoglobin after intravenous administration of some alkylanilines*

| Compound | No. of cats | Dose mmol /kg | Methaemoglobin % ± s.e. after time: 1 h | 2 h | 3 h | 4 h | 5 h | Mean | Mean max. |
|---|---|---|---|---|---|---|---|---|---|
| 2-Methylaniline .. | 9 | 0·25 | 57·4 | 63·9 | 64·5 | 63·3 | 57·1 | 61·3 | 70·1 |
| | | | 3·8 | 3·4 | 3·6 | 4·9 | 3·9 | | 3·9 |
| 3-Methylaniline .. | 8 | 0·25 | 28·8 | 46·8 | 57·5 | 60·8 | 58·6 | 50·5 | 60·2 |
| | | | 2·9 | 5·2 | 6·1 | 6·5 | 6·6 | | 6·5 |
| 4-Methylaniline .. | 9 | 0·25 | 28·1 | 34·3 | 32·7 | 33·2 | 32·1 | 32·1 | 39·6 |
| | | | 5·3 | 5·1 | 4·7 | 4·6 | 5·0 | | 4·8 |
| 2,3-Dimethylaniline .. | 5 | 0·25 | 16·9 | 16·3 | 15·2 | 13·4 | 9·5 | 14·3 | 20·2 |
| | | | 4·8 | 5·5 | 6·0 | 6·1 | 4·0 | | 5·4 |
| 2,4-Dimethylaniline .. | 15 | 0·25 | 6·6 | 6·5 | 7·6 | 6·9 | 3·7 | 6·3 | 10·4 |
| | | | 1·8 | 2·2 | 2·9 | 2·8 | 2·1 | | 3·7 |
| 2,5-Dimethylaniline .. | 5 | 0·25 | 30·7 | 35·2 | 30·0 | 28·6 | 21·1 | 29·1 | 36·3 |
| | | | 5·0 | 7·7 | 4·6 | 2·6 | 4·3 | | 7·1 |
| 2,6-Dimethylaniline .. | 5 | 0·25 | 8·7 | 8·6 | 7·6 | 6·3 | 4·8 | 7·2 | 10·3 |
| | | | 2·6 | 2·1 | 1·8 | 1·8 | 1·2 | | 1·9 |
| 3,4-Dimethylaniline .. | 5 | 0·25 | 11·8 | 14·1 | 15·6 | 16·6 | 12·2 | 14·1 | 18·0 |
| | | | 2·1 | 2·5 | 3·1 | 3·7 | 3·5 | | 3·4 |
| 3,5-Dimethylaniline .. | 5 | 0·25 | 44·4 | 41·9 | 38·4 | 35·9 | 31·5 | 38·3 | 46·5 |
| | | | 3·0 | 5·3 | 3·5 | 4·0 | 4·9 | | 3·9 |
| 2-Ethylaniline .. .. | 5 | 0·0625 | 26·0 | 21·3 | 13·4 | 9·2 | 9·8 | 15·9 | 27·1 |
| | | | 4·4 | 2·9 | 2·8 | 1·5 | 1·6 | | 4·1 |
| 3-Ethylaniline .. .. | 5 | 0·0625 | 20·6 | 23·2 | 25·0 | 23·9 | 22·1 | 23·0 | 28·3 |
| | | | 2·8 | 3·7 | 5·1 | 4·2 | 4·4 | | 4·3 |
| 4-Ethylaniline .. .. | 5 | 0·0625 | 38·0 | 61·0 | 64·8 | 61·2 | 56·9 | 56·4 | 66·7 |
| | | | 7·4 | 3·6 | 5·2 | 5·0 | 4·6 | | 4·5 |

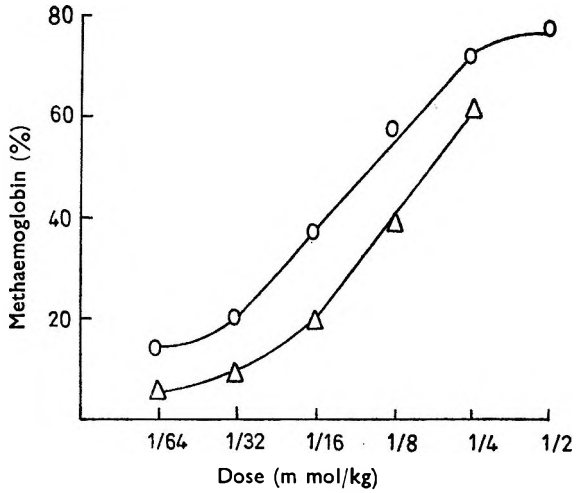


FIG. 2. The relationship between log dose of 3-methylaniline (△), aniline (○) and the maximum methaemoglobin response. 3-Methylaniline was given to 8 cats at 0·25 mmole/kg and to 2 cats at each other dose. Aniline was given to at least 5 cats at each dose level.

were approximately parallel (Fig. 2). Pretreatment with SKF 525A (60 mg/kg i.p. 45 min before administration of the amine) had little effect on methaemoglobin formation by 2-, 3- and 4-methylaniline. All the dimethylanilines formed less methaemoglobin than aniline (Table 4). The most active were 2,5- and 3,5-dimethylaniline. A Student's *t*-test showed no significant difference in the maximum methaemoglobin responses to 2,3-, 2,4- 2,5- and 3,4-dimethylaniline. 2,4,6-Trimethylaniline formed no methaemoglobin. The level of methaemoglobin formed by the dimethylanilines remained relatively constant over 5 h. 2,4-Dimethylaniline (0·25 mmol/kg i.v.) was given to 15 cats and the frequency distribution of the maximum responses was found to be skewed, with more than half the animals forming less than 5% methaemoglobin (Fig. 3).

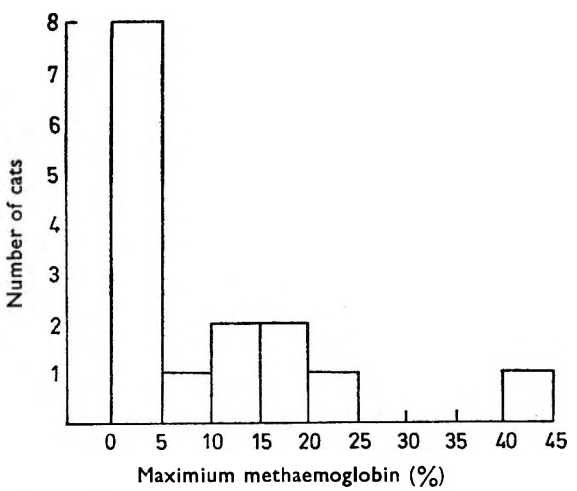


FIG. 3. Frequency distribution of the maximum methaemoglobin response in cats after intravenous administration of 2,4-dimethylaniline at 0·25 mmol/kg.

Ethyl groups in the 2- and 3-positions slightly reduced the methaemoglobin-forming activity of aniline ($P < 0.05$) while 4-ethylaniline was much more active than aniline (Table 4). The response to 4-ethylaniline was maximal at the third hour.

*Alkoxy, carbinol and carboxy substituted anilines.* The anisidines (2-, 3- and 4-) were all less active than aniline (Table 5). The maximum responses to 3- and 4-phenetidine were not significantly different from that to aniline, possibly because the standard errors for these phenetidines were large. Aniline was more active than 2-phenetidine. The methaemoglobin responses to the anisidines and phenetidines declined relatively rapidly. 4-Aminobenzylalcohol was slightly less active than aniline, while 2-aminobenzylalcohol was very much less active and 3-aminobenzylalcohol was virtually inactive. Benzocaine and procaine, two esters of 4-aminobenzoic acid, were much less active than aniline. The response to the aminobenzylalcohols, benzocaine and procaine fell rapidly from 1 h to 5 h. The aminobenzoic acids (2-, 3-, and 4-) did not form detectable amounts of methaemoglobin.

Table 5. *Formation of methaemoglobin after intravenous administration of some substituted anilines.* Five cats were used in each experiment, except for benzocaine where one animal was used.

| Compound | Dose mmol /kg | Methaemoglobin % ± s.e. after time: 1 h | 2 h | 3 h | 4 h | 5 h | Mean | Mean max. |
|---|---|---|---|---|---|---|---|---|
| 2-Anisidine .. .. | 0·0625 | 11·5 | 6·5 | 3·9 | 3·2 | 1·0 | 5·2 | 11·5 |
| | | 3·4 | 2·8 | 2·1 | 1·6 | 0·6 | | 3·4 |
| 3-Anisidine .. .. | 0·0625 | 20·1 | 16·1 | 12·6 | 8·5 | 6·9 | 12·8 | 20·1 |
| | | 5·5 | 5·3 | 4·2 | 4·1 | 4·0 | | 5·5 |
| 4-Anisidine .. .. | 0·0625 | 13·4 | 15·3 | 7·6 | 3·5 | 1·6 | 8·3 | 15·3 |
| | | 2·3 | 2·6 | 1·7 | 1·2 | 0·7 | | 2·6 |
| 2-Phenetidine .. | 0·0625 | 7·3 | 5·0 | 2·7 | 1·7 | 0·7 | 3·9 | 7·8 |
| | | 3·1 | 3·2 | 0·8 | 0·6 | 0·2 | | 2·9 |
| 3-Phenetidine .. .. | 0·0625 | 21·7 | 25·0 | 15·6 | 12·1 | 7·2 | 16·3 | 27·5 |
| | | 4·6 | 8·2 | 4·9 | 3·6 | 2·7 | | 7·9 |
| 4-Phenetidine .. .. | 0·0625 | 37·6 | 46·7 | 39·0 | 27·6 | 20·1 | 34·2 | 46·7 |
| | | 9·3 | 9·9 | 8·8 | 8·4 | 6·7 | | 9·9 |
| 2-Aminobenzylalcohol | 0·25 | 10·7 | 10·0 | 6·9 | 4·3 | 2·4 | 6·9 | 11·0 |
| | | 2·9 | 2·9 | 2·4 | 1·7 | 1·1 | | 2·8 |
| 3-Aminobenzylalcohol | 0·25 | 1·2 | 0·8 | 0·3 | 0·6 | 0·3 | 0·6 | 1·4 |
| | | 0·6 | 0·2 | 0·2 | 0·2 | 0·2 | | 0·5 |
| 4-Aminobenzylalcohol | 0·0625 | 26·8 | 17·4 | 12·9 | 7·8 | 6·8 | 14·4 | 26·8 |
| | | 3·7 | 3·9 | 3·2 | 1·7 | 2·0 | | 3·7 |
| Benzocaine .. .. | 0·25 | 21·7 | 13·4 | 9·8 | 3·5 | 2·0 | 10·1 | 21·7 |
| Procaine .. .. | 0·20 | 6·7 | 10·4 | 9·5 | 7·1 | | 8·4 | 13·1 |
| | | 1·6 | 2·1 | 3·8 | 3·0 | | | 3·3 |

## DISCUSSION

*Methaemoglobin in untreated cats.* As the normal level of methaemoglobin in a population of 152 cats was 1·1%, less than the limit of sensitivity of the assay, it was taken as being equal to zero in subsequent experiments.

*Irreversible destruction of haemoglobin.* Methaemoglobin formation has frequently been found to be associated with the oxidative destruction of haemoglobin and the appearance of irreversible degradation products including sulphaemoglobin and Heinz bodies (Allen & Jandl, 1961; Harley & Mauer, 1960). There is also doubt, however, (Beutler, 1962; Rentsch, 1968) about whether a causal relation exists between methaemoglobin formation and haemoglobin destruction. Cat haemoglobin is known to be resistant to oxidative denaturation (Robin & Harley, 1966). Nevertheless it was

considered necessary to determine the degree of haemoglobin destruction in these experiments since the accuracy of the methaemoglobin estimations would be materially affected if such destruction was extensive. A simple Heinz body count in red cells would not have been satisfactory since similar inclusions have been reported in the red cells of normal cats (Schmauch, 1899) with extreme frequency variations (Beritic, 1965). More meaningful information was expected from measurements of "intact haemoglobin" remaining after the formation of methaemoglobin by drugs. After several experiments in which the methaemoglobin responses to a number of aromatic amines were followed for several hours the average "intact haemoglobin" concentration had fallen by only 5% from the initial value. There may have been considerable turnover of red cells with damaged cells being sequestered and new cells released by the reticulo-endothelial system (Kiese & Kaeske, 1942; Rothberg, Corallo & Crosberg, 1959), but this could not be detected by the techniques used. It was concluded that the estimation of methaemoglobin under these conditions was not greatly affected by irreversible destruction of haemoglobin.

*Formation of methaemoglobin by aniline.* It has been shown (McLean & others, 1967) that the methaemoglobin response to aniline has an approximately normal distribution and that the response is reproducible. A linear dependence of the methaemoglobin response to aniline on log dose was also found. It can be seen from Table 2 that at a high dose level (0·25 mmol/kg) the response was greater after intravenous than after oral administration, but at a lower dose (0·0625 mmol/kg) there was no significant difference except that the initial rate of formation of methaemoglobin was slower when the oral route was used.

After intravenous administration of aniline (0·0625 mmol/kg) the level of methaemoglobin rose steeply almost at once (Fig. 1). The response levelled out at a maximum between 1 and 2 h after the dose had been given, and then declined fairly steadily until at 5 h it was less than half the maximum value. The formation of methaemoglobin could therefore be followed by estimating methaemoglobin levels hourly for 5 h after administration of aniline.

*Methaemoglobin formation by substituted anilines*

Most aromatic amines induce methaemoglobin formation in the intact animal but not on incubation with blood or red cell suspensions (Bodansky, 1951). Metabolism to the molecular species capable of reacting directly with haemoglobin and oxygen, such as aminophenols and *N*-oxidation products, occurs mainly in the liver (Kiese, 1966). Phenylhydroxylamine (von Issekutz, 1939) and nitrosobenzene (Kiese & Soetbeer, 1950) have been shown to be the most effective metabolites of aniline in producing methaemoglobin *in vivo*. Aminophenols are generally considered to be of minor importance (Kiese, 1966) although some arylamines form highly active aminophenols (Kiese & Rachor, 1964).

Aniline substituted with -Et, -OEt, -$CH_2OH$, -Cl, -Br, or -F was most active if the substituents were in the 4-position and less active if 3-substituted. The activity of the 2-isomer was either less than or equal to that of the 3-isomer. Exceptions were the -Me and -OMe groups, which produced less activity in the 4-position than in the 3-position. These findings are supported by the observations of Kiese (1963) that 4-substitution of aniline by -Cl, alkoxy or acyl residues increased methaemoglobin formation in dogs while the same substituents in the 2- or 3-positions decreased activity. The exception, as in the present study, was 4-methylaniline which formed less

methaemoglobin than either its 2- and 3-isomers, or aniline. Anaesthesia reduces the formation of methaemoglobin by aromatic amines to a marked extent (McLean & others, 1967). Although Kiese (1963) used anaesthetized dogs (50 mg chloralose + 500 mg urethane/kg, i.p.) the order of activities of the substituted anilines was the same as in the present study, where the cats were unanaesthetized.

Kiese (1966) found that 4-substitution of aniline frequently increased the rate of microsomal *N*-hydroxylation, and von Jagow, Kiese & Renner (1966) observed that 4-substitution of aniline favoured the urinary excretion of *N*-hydroxy derivatives. It was suggested that the substituents were blocking 4-hydroxylation, a major metabolic reaction of aromatic amines which presumably competes with *N*-hydroxylation. It has been shown, however, that cats and dogs hydroxylated aniline mainly in the 2-position (Parke, 1960) and therefore any increase in *N*-hydroxylation due to blocking of the preferred site of ring hydroxylation would be expected with 2-substituted amines. This did not occur. Furthermore, rats, which hydroxylated aniline mainly in the 4-position, excreted as ring-hydroxylated metabolites at least as much after a dose of 4-chloroaniline (62%) as after 2-chloroaniline (54%) (Newell, Argus & Ray, 1960). It is therefore unlikely that the blocking of 4-hydroxylation accounts for the increased *N*-hydroxylation seen in 4-substituted anilines. Other factors, possibly related to enzyme fit, must be involved.

The high methaemoglobin-forming activity of 4-substituted anilines, and the low activity of the 2- and 3-isomers, may reflect the activities of their *N*-hydroxy derivatives. Despite a high rate of *N*-hydroxylation by dog liver microsomes, 3-aminopropiophenone formed little methaemoglobin *in vivo* because 3-hydroxylaminopropiophenone reacted very slowly with haemoglobin and was consumed in side reactions (Kiese & Rauscher, 1965). On the other hand, 4-aminopropiophenone was more rapidly *N*-hydroxylated than aniline and 4-hydroxylaminopropiophenone oxidized haemoglobin in the red cell at three times the rate of phenylhydroxylamine (Graffe, Kiese & Rauscher, 1964).

Steric factors did not seem to be very important in the activities of most of the substituted anilines tested. Although 2-Et, -OEt and -OMe anilines formed less methaemoglobin than the 3-isomers this is unlikely to be due to steric retardation of activity, since this effect was absent with the equally bulky -Cl, -Br and Me groups and there was no difference in the activity of 2,4- and 2,6-dimethylaniline. However, 2,6-dibromo- and 2,4,6-tribromoaniline were less active than the corresponding di- and trichloroanilines. This was presumably due to steric effects since the monobromoanilines were as active as the monochloroanilines.

The situation may be complicated by the metabolic reactions of the substituent groups. Replacement of chlorine (Betts, Bray & others, 1957) or fluorine (Renson, 1964; Daly, Guroff & others, 1968) in the 4-position of aromatic amines by hydroxyl has been reported as a minor metabolic pathway. This reaction is not extensive enough to account for e.g. the difference in the activities of 4-chloro- and 4-fluoroaniline. There was no evidence for hydroxylation-induced migration of halogen from the 4- to the 3-position ("The NIH Shift"; Guroff, Daly & others, 1967) in 4-fluoro- or chloroaniline or the corresponding acetanilides incubated with rabbit liver microsomes (Daly & others, 1968). Although metabolically very stable, the halogen substituents may affect the activity of the amine by blocking ring hydroxylation. For example, it is possibly because of the blocking of all the major sites of ring hydroxylation that 2,4,6-trichloro- and 2,4,6-tribromoaniline formed more methaemoglobin than the

corresponding 2,5-dihaloanilines. Alternatively, this may be simply due to the high activity associated with a -Cl or -Br in the 4-position.

Aromatic alkyl groups are oxidized *in vivo via* the alcohol to the carboxylic acid (Williams, 1959). 4-Acetotoluidide was oxidized to the carboxylic acid, whereas ring hydroxylation was the preferred metabolic reaction for the 2- and 3-acetotoluidides (Bray & Thorpe, 1948). Rapid conversion to the inactive aminobenzoic acid may be thought to explain the low activity of 4-methylaniline. However, it has been reported that after intravenous administration of equimolar doses of aniline and 2-, 3- and 4-methylaniline to dogs, the blood concentration of the amines fell at the same rate over 5 h and that 4-methylaniline produced a higher blood level of the *N*-hydroxy derivative than was found after aniline (Kiese 1963). Moreover, the methaemoglobin response to 2-, 3- and 4-methylaniline was unaffected by pretreatment with SKF 525 A [2-(diethylamino)ethyl-2,2-diphenylvalerate hydrochloride], a compound known to inhibit the oxidation of alkyl side-chains (Cook, Tonor & Fellows, 1954). Thus the different activities of the methylanilines seem to be related to differences in the intrinsic activities of the *N*-hydroxy compounds.

Daly & others (1968) found that 4-ethylacetanilide was metabolized mainly to 4-(1′-hydroxyethyl)acetanilide by rabbit liver microsomes. Since further oxidation of alcohols to ketones or aldehydes is thought to be due to the alcohol dehydrogenase in the soluble fraction of liver (Gillette, 1959), the possible formation of the *N*-hydroxy metabolite of 4-aminoacetophenone from 4-ethylaniline may explain the high activity of 4-ethylaniline over its 2- and 3-isomers. A similar mechanism, involving the *N*-hydroxy metabolites of the aminobenzaldehydes, may account for the high activity of 4-aminobenzylalcohol compared with its 2- and 3-isomers.

4-Phenetidine was much more active than 4-anisidine. This difference is not related to a difference in the rates of ether cleavage to 4-aminophenol since 4-OEt and 4-OMe acetanilide were cleaved at the same rate by rabbit liver microsomes (Axelrod, 1956). However, aminophenol metabolites may account for some of the activity of the alkoxy anilines since in the cat, 4-aminophenol was approximately as active as aniline, and 2-aminophenol was more active in forming methaemoglobin (Kiese & Rachor, 1964).

The low activities of benzocaine and procaine are likely to be due to their rapid hydrolysis *in vivo* to the inactive 4-aminobenzoic acid (Williams, 1959). Aminobenzoic acids, being ionized as zwitter ions, presumably do not reach the sites of *N*-hydroxylation in the endoplasmic reticulum (Brodie, 1964). The very weak activity of the polyfluoroanilines is possibly due to their low water solubility, and therefore poor absorption (Brodie & Hogben, 1957). 2,4,6-Trimethylaniline confirmed the trend that increasing the number of methyl substitutents diminishes methaemoglobin formation by aniline, possibly because the *N*-hydroxy derivatives are less active.

*Acknowledgments*

One of us (S. M.) wishes to thank the Pharmaceutical Society of New South Wales S. E. Wright Research Trust for a research grant. SKF 525A was supplied by Smith Kline and French Pty. Ltd. Some fluoroanilines were supplied by Dr. F. Rose of ICI.

REFERENCES

ALLEN, D. W. & JANDL, J. H. (1961). *J. clin. Invest.*, **40**, 454–475.
AXELROD, J. (1956). *Biochem. J.*, **63**, 634–639.
BERITIC, T. (1965). *Blood*, **15**, 999–1008.

BETTS, J. J., BRAY, H. G., JAMES, S. P. & THORPE, W. V. (1957). *Biochem. J.*, **66**, 610–621.

BEUTLER, E. (1962). *Nature, Lond.*, **196**, 1095–1096.

BODANSKY, O. (1951). *Pharmac. Rev.*, **3**, 144–196.

BRAY, H. G. & THORPE, W. V. (1948). *Biochem. J.*, **43**, 211–216.

BRODIE, B. B. (1964). *Physico-chemical factors in drug absorption, in Absorption and Distribution of Drugs.* Editor: T. B. Binns, p. 15–48, London: Livingstone.

BRODIE, B. B. & HOGBEN, A. M. (1957). *J. Pharm. Pharmac.*, **9**, 345–380.

COOK, L., TONOR, J. J. & FELLOWS, E. T. (1954). *J. Pharmac. exp. Ther.*, **111**, 131–141.

DALY, J. W., GUROFF, G., UDENFRIEND, S. & WITKOP, B. (1968). *Biochem. Pharmac.*, **17**, 31–36.

GILLETTE, J. R. (1959). *J. biol. Chem.*, **234**, 139–143.

GRAFFE, W., KIESE, M. & RAUSCHER, E. (1964). *Arch. exp. Path. Pharmak.*, **249**, 168–175.

GUROFF, G., DALY, J. W., JERINA, D. M. RENSON, J., WITKOP, B. & UDENFRIEND, S. (1967). *Science*, N.Y., **157**, 1524–1530.

HARLEY, J. D. & MAUER, A. M. (1960). *Blood*, **16**, 1722–1735.

VON ISSEKUTZ, B. V. (1939). *Arch. exp. Path. Pharmak.*, **193**, 551–566.

VON JAGOW, R., KIESE, M. & RENNER, G. (1966). *Biochem. Pharmac.*, **15**, 1899–1910.

KIESE, M. (1963). *Arch. exp. Path. Pharmak.*, **244**, 387–404.

KIESE, M. (1966). *Pharmac. Rev.*, **18**, 1091–1161.

KIESE, M. & KAESKE, H. (1942). *Biochem. Z.*, **312**, 121–149.

KIESE, M. & RACHOR, M. (1964). *Arch. exp. Path. Pharmak.*, **249**, 225–236.

KIESE, M. & RAUSCHER, E. (1965). *Ibid.*, **251**, 201–202.

KIESE, M. & SOETBEER, M. (1950). *Ibid.*, **210**, 305–310.

LESTER, D. (1943). *J. Pharmac. exp. Ther.*, **77**, 154–159.

MCLEAN, S. (1968). Ph.D. Thesis, University of Sydney, pp. 87–111.

MCLEAN, S., MURPHY, B. P., STARMER, G. A. & THOMAS, J. (1967). *J. Pharm. Pharmac.*, **19**, 146–154.

MCLEAN, S., ROBINSON, J., STARMER, G. A. & THOMAS, J. (1967). *Ibid.*, **19**, 803–809.

MÜLLER-OERLINGHAUSEN, B. & BAETHKE, R. (1966). *Arch. exp. Path. Pharmak.*, **253**, 327–335.

NEWELL, M., ARGUS, N. & RAY, F. E. (1960). *Biochem. Pharmac.*, **5**, 30–38.

PARKE, D. V. (1960). *Biochem. J.*, **77**, 493–503.

RENSON, J. (1964). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **23**, 325.

RENTSCH, G. (1968). *Biochem. Pharmac.*, **17**, 423–427.

ROBIN, H. & HARLEY, J. D. (1964). *Australas. Ann. Med.*, **13**, 313–319.

ROBIN, H. & HARLEY, J. D. (1966). *Aust. J. exp. Biol. med. Sci.*, **44**, 519–526.

ROTHBERG, H., CORALLO, L. A. & CROSBERG, W. H. (1959). *Blood*, **14**, 1180–1186.

SCHMAUCH, G. (1899). *Arch. Anat. Physiol.*, **156**, 201–244.

SPICER, S. S. (1950). *J. Pharmac. exp. Ther.*, **99**, 185–194.

WILLIAMS, R. T. (1959). *Detoxication Mechanisms*, 2nd edn, London: Chapman Hall.

*J. Pharm. Pharmac.*, 1969, **21**, 451–459 *Received January* 13, 1969

# The effect of anti-inflammatory drugs on the protein-binding of 11-hydroxysteroids in human plasma *in vitro*

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The effect of acetylsalicylic acid, ibufenac, indomethacin, oxyphenbutazone and phenylbutazone on the protein-binding of 11-hydroxysteroids *in vitro* at concentrations in excess of normal has been examined in human blood bank plasma, in plasma from women in the third trimester of pregnancy, and in plasma from patients after injections of tetracosactide. Phenylbutazone, ibufenac and indomethacin do not significantly affect the protein-binding of 11-hydroxysteroids. Oxyphenbutazone causes a decrease in the proportion of unbound 11-hydroxysteroids and acetylsalicylic acid produces an increase of unbound 11-hydroxysteroids.

We have already shown that acetylsalicylic acid, phenylbutazone, and indomethacin in therapeutic doses do not significantly affect the binding of 11-hydroxysteroids to plasma proteins in humans with rheumatoid arthritis (Stenlake, Davidson & others, 1968). The total concentration of 11-hydroxysteroids in all these patients was well within the normal physiological range (6·5—26·3 $\mu$g/100 ml, Mattingly, 1962), and in consequence the levels of unbound 11-hydroxysteroids were such that in some determinations they came near the limiting concentration detectable by the spectrophotofluorimetric method used. It seemed advisable, therefore, to examine the effect of acetylsalicylic acid and other anti-inflammatory drugs on the protein-binding of 11-hydroxysteroids at higher total steroid concentrations, including concentrations in excess of the normal corticosteroid binding capacity (CBC) of plasma transcortin which was found by De Moor, Heirwegh & others (1962) to be 26 $\pm$ 3·8 $\mu$g/100 ml of plasma.

Endogenous levels of 11-hydroxysteroids in human plasma exceed the normal CBC only in abnormal physiological states, such as in Cushing's syndrome and in women in the third trimester of pregnancy, when they frequently rise to two or three times normal values. They are similarly raised after the administration of corticotrophin, and tetracosactide (Synacthen), a synthetic 1–24 polypeptide having adrenocortical stimulating properties identical to those of corticotrophin. Since, however, only a few such patients could be made available to extend our studies of the effect of aspirin and other anti-inflammatory drugs on the plasma protein-binding of 11-hydroxysteroids, we have also used *in vitro* systems in which the 11-hydroxysteroid concentrations have been artificially raised, with the object of extrapolating our results back to normal physiological levels.

## EXPERIMENTAL

### *Materials*

In addition to those already described (Stenlake & others, 1968) the following drugs were used; indomethacin (Merck Sharp & Dohme Ltd.); oxyphenbutazone (Geigy); ibufenac (Boots Pure Drug Co. Ltd.); albumin, human lyophilized (Kabi); Depot Synacthen injection (Ciba).

*Collection of blood and ultrafiltration of plasma*

Plasma (65 ml) was obtained from human blood as previously described (Stenlake & others, 1968). In some experiments plasma from the blood-bank was used.

Ultrafiltration of the plasma or albumin solutions was carried out at 37° by the method of Toribara (1953) as previously reported (Stenlake & others, 1968).

*Measurement of* 11-*hydroxysteroids*

*Standard procedure.* The concentration of 11-hydroxysteroids in plasma samples and ultrafiltrates was determined by the spectrofluorimetric method of Mattingly (1962), except that the number of test solutions in each batch was increased to six.

*Protein-binding*

1. *Effect of anti-inflammatory drugs on the binding of* 11-*hydroxysteroids to the proteins in plasma from patients with rheumatoid arthritis.* Plasma (65 ml) was obtained from each of six rheumatoid arthritic patients treated with a placebo, calcium lactate (600 mg) in tablet form for four days, to ensure that the clinical effect of residual anti-inflammatory drugs from previous therapy was minimal. The level of 11-hydroxysteroids in each plasma sample was increased by 50 $\mu$g/100 ml by adding the plasma to the dry residue of standard hydrocortisone solution (0·65 ml; 50 $\mu$g/ml in 5% aqueous ethanol). Equilibration of the 11-hydroxysteroids between the protein-bound and the unbound forms was achieved by incubation of the plasma for 60 min at 37°. Aliquots (10 ml) were added to each of six stoppered tubes, one without drug, and the others containing respectively acetylsalicylic acid (10 mg), ibufenac (1 mg), indomethacin (0·2 mg), oxyphenbutazone (2 mg) and phenylbutazone (2 mg). After being shaken overnight, the tubes were centrifuged briefly to disperse the froth formed and each aliquot was then ultrafiltered. The concentration of 11-hydroxysteroids in the plasma samples, ultrafiltrates and controls was determined in duplicate. The results are in Table 1.

2. *Effect of acetylsalicylic acid on the binding of* 11-*hydroxysteroids to proteins of normal, rheumatoid arthritic and blood-bank plasma.* Plasma samples (65 ml), obtained from (a) seven healthy volunteers, (b) a further seven rheumatoid arthritic patients who had received no anti-inflammatory drug in the seven days before the withdrawal of blood, and (c) the blood-bank (four different samples) were similarly equilibrated first with hydrocortisone (50 $\mu$g/100 ml), and then with acetylsalicylic acid (100 mg/100 ml) and ultrafiltered. Concentrations of 11-hydroxysteroids in the plasma samples, ultrafiltrates and controls were determined in duplicate. The results are in Table 2.

3. *Effect of different concentrations of acetylsalicylic acid on the binding of* 11-*hydroxysteroids to proteins in blood-bank plasma.* The concentration of 11-hydroxysteroids in blood-bank plasma was increased by 50 $\mu$g/100 ml, as described in experiment 1. Aliquots were equilibrated with acetylsalicylic acid to give final concentrations of 20, 40, 60, 80 and 100 mg/100 ml respectively and ultrafiltered. Concentrations of 11-hydroxysteroids in the plasma, ultrafiltrates and controls were determined in duplicate. The results are in Fig. 1.

4. *Effect of acetylsalicylic acid on the protein-binding of* 11-*hydroxysteroids in blood-bank plasma at different total concentrations of* 11-*hydroxysteroids.* The concentration of 11-hydroxysteroids in six samples of the same blood-bank plasma was increased by 0, 5, 10, 20, 30 and 50 $\mu$g/100 ml respectively, as described in experiment 1. Each sample was divided into two equal portions, one of which was equilibrated with

acetylsalicylic acid (100 mg/100 ml) and ultrafiltered. Concentrations of 11-hydroxysteroids present in the ultrafiltrates and controls were determined in triplicate. The results are in Table 3.

5. *Effect of incubation time with acetylsalicylic acid on the displacement of* 11-*hydroxysteroids from their protein-binding sites in blood-bank plasma.* The concentration of 11-hydroxysteroids in a sample of blood-bank plasma was increased by 50 $\mu$g/100 ml, as described in experiment 1, and acetylsalicylic acid (100 mg/100 ml) dissolved in each of seven fractions. Ultrafiltration of the fractions was begun 0·5, 1, 2, 5, 10, 20 and 50 h respectively after the addition of the acetylsalicylic acid, and concentrations of 11-hydroxysteroids in the control and in each ultrafiltrate were determined in duplicate.

6. *Effect of acetylsalicylic acid on the protein-binding of* 11-*hydroxysteroids in plasma in which the endogenous level of* 11-*hydroxysteroids is high.* Plasma (60 ml) was obtained from two rheumatoid arthritic patients 4 h after an intramuscular injection of tetracosactide (Depot Synacthen) and from two normal, pregnant women, both within one week of delivery. All these patients had received no anti-inflammatory drugs in the seven days before the withdrawal of blood. Acetylsalicylic acid (100 mg/100 ml) was dissolved in samples and each plasma aliquot was ultrafiltered. Concentrations of 11-hydroxysteroids in the ultrafiltrates and controls were measured in duplicate. The results are in Table 5.

7. *Effect of acetylsalicylic acid on the binding of* 11-*hydroxysteroids to human serum albumin.* A solution of serum albumin (4% in 0·1M phosphate buffer, pH 7·4) was prepared and added to the dry residue of standard hydrocortisone solution (50 $\mu$g/ml) to give a concentration of 25 $\mu$g/ml. After equilibration (60 min at 37°) acetylsalicylic acid (100 mg/100 ml) was added to an aliquot, the solution further equilibrated and ultrafiltered. Concentrations of 11-hydroxysteroids in treated and control ultrafiltrates were measured in duplicate.

8. *Effect of anti-inflammatory drugs on the spectrophotofluorimetric determination of* 11-*hydroxysteroids.* The following drugs were examined as described at the concentrations stated for their effect on the measurement of 11-hydroxysteroid fluorescence.

(a) *Ibufenac* (10 mg/100 ml), dissolved in a standard hydrocortisone solution (20 $\mu$g/100 ml). The apparent concentration of 11-hydroxysteroids was compared with the concentration of the same standard solution without added drug.

(b) *Acetylsalicylic acid.* The 18 plasma samples examined in experiment 2 were assayed for 11-hydroxysteroids before and after the addition of acetylsalicylic acid (100 mg/100 ml).

(c) *Oxyphenbutazone* (20 mg/100 ml), dissolved in a standard solution of hydrocortisone (20 $\mu$g/100 ml), was examined as under (a).

Blood-bank plasma with the 11-hydroxysteroids artificially increased by 50 $\mu$g/100 ml was assayed for 11-hydroxysteroids before and after the addition of oxyphenbutazone (20 mg/100 ml).

Oxyphenbutazone (1·5 mg/100 ml*) was also added to an ultrafiltrate sample from blood-bank plasma in which the 11-hydroxysteroids had been artificially increased by 50 $\mu$g/100 ml, and the solution examined as under (a).

(d) *Phenylbutazone* (20 mg/100 ml), dissolved in standard hydrocortisone solution (20 $\mu$g/100 ml), was examined as under (a).

* The concentration of oxyphenbutazone in an ultrafiltrate of plasma containing 20 mg/100 ml was found by the method of Burns, Rose & others (1955) to be 1·5 mg/100 ml.

9. *Effect of the anti-inflammatory drugs on plasma pH.* The concentration of 11-hydroxysteroids in blood-bank plasma and rheumatoid arthritic plasma was increased by 50 $\mu$g/100 ml as described in experiment 1. Acetylsalicylic acid (100 mg/100 ml), ibufenac (10 mg/100 ml), indomethacin (2 mg/100 ml), oxyphenbutazone (20 mg/100 ml) and phenylbutazone (20 mg/100 ml) were added to separate samples (10 ml) of each plasma. The pH values of the plasma samples and controls were measured.

10. *Effect of pH on the binding of 11-hydroxysteroids to plasma proteins.* Hydrochloric acid (N/2 about 0·2 ml) was added to a further portion of each plasma used in experiment 9 to give the same pH as that produced by the acetylsalicylic acid. The samples containing acetylsalicylic acid and each control were diluted with demineralized water to the same volume as the corresponding samples with hydrochloric acid, and ultra-filtered. Concentrations of 11-hydroxysteroids in each were measured in duplicate.

## RESULTS AND DISCUSSION

The use of *in vitro* systems permitted experiments with plasma concentrations of steroids and anti-inflammatory drugs well in excess of those normally found in human plasma after administration of therapeutic doses of the drugs. Drugs were used at plasma concentrations four times those normally found after therapeutic doses (Smith, Gleason & others, 1946; Yu, Burns & others, 1958; Rechenberg & Herrmann, 1961; Holt & Hawkins, 1965; Adams, S. S. & Cliff, E. E., personal communication) and 11-hydroxysteroid levels were raised in most experiments by the addition of hydrocortisone (50 $\mu$g/100 ml) giving plasma total 11-hydroxysteroid levels of 55–70 $\mu$g/100 ml. For these reasons, it was necessary to check for quenching of fluorescence at the drug concentrations used.

Ibufenac (10 mg/100 ml) and phenylbutazone (20 mg/100 ml) did not interfere with the fluorimetric determination of 11-hydroxysteroids. Both acetylsalicylic acid and oxyphenbutazone, however, caused slight quenching of fluorescence of 11-hydroxysteroids *in vitro* and in plasma. Thus the mean quenching of 11-hydroxysteroid fluorescence in eighteen different plasma samples containing on average 60·6 $\mu$g/100 ml of steroid (experiment 2) was 1·82 $\pm$ s.d. 0·63 $\mu$g/100 ml after the addition of acetylsalicylic acid (100 mg/100 ml). Since acetylsalicylic acid is appreciably bound to plasma proteins, its concentrations in ultrafiltrates are much less than those in plasma. Concentrations of 11-hydroxysteroids in plasma ultrafiltrates containing acetylsalicylic acid are, therefore, reported uncorrected for this slight quenching effect.

Table 1. *Effect of acetylsalicylic acid, ibufenac, indomethacin, oxyphenbutazone and phenylbutazone on the protein-binding of 11-hydroxysteroids in plasma from rheumatoid arthritic patients*

| | | Unbound 11-hydroxysteroids $\mu$g/100 ml | | | | | |
|---|---|---|---|---|---|---|---|
| Patient | Total plasma 11-hydroxysteroids $\mu$g/100 ml | Control | Acetylsalicylic acid | Ibufenac | Indomethacin | Oxyphenbutazone | Phenylbutazone |
| 1 | 62·0 | 17·6 | 23·9 | 18·8 | 17·3 | 12·9 | 16·9 |
| 2 | 60·4 | 14·5 | 20·0 | 15·3 | 14·5 | 12·2 | 14·5 |
| 3 | 65·1 | 16·9 | 22·4 | 16·1 | 16·9 | 13·7 | 16·9 |
| 4 | 65·8 | 19·2 | 23·9 | 18·8 | 20·8 | 14·5 | 18·8 |
| 5 | 58·7 | 15·7 | 21·6 | 15·3 | 15·7 | 12·2 | 15·3 |
| 6 | 61·8 | 14·5 | 17·3 | 13·7 | 14·5 | 11·0 | 13·7 |
| Mean $\pm$ s.d. .. | 62·3 $\pm$ 2·72 | 16·4 $\pm$ 1·86 | 21·5 $\pm$ 2·54 | 16·3 $\pm$ 2·06 | 16·6 $\pm$ 2·36 | 12·8 $\pm$ 1·24 | 16·0 $\pm$ 1·87 |
| % Unbound mean $\pm$ s.d. .. .. | | 26·3 $\pm$ 2·29 | 34·5 $\pm$ 3·71 | 26·2 $\pm$ 2·89 | 26·6 $\pm$ 2·94 | 20·4 $\pm$ 1·44 | 25·7 $\pm$ 2·30 |
| Student's $t$ .. | | | 3·95 | 0·089 | 0·163 | 3·96 | 0·361 |
| $P$ .. .. .. | | | <0·01 | >0·9 | >0·8 | <0·01 | >0·7 |

Quenching of fluorescence by oxyphenbutazone similarly reduced actual 11-hydroxysteroid concentrations of 20 μg/100 ml in standard hydrocortisone solution, and 65·6 μg/100 ml in plasma to apparent concentrations of 18 and 63·8 μg/100 ml respectively. However, plasma containing oxyphenbutazone (20 mg/100 ml) gave ultrafiltrates containing only 1·5 mg/100 ml, and addition of oxyphenbutazone (1·5 mg/100 ml) to an aliquot of an ultrafiltrate from plasma in which the 11-hydroxysteroids had been increased by 50 μg/100 ml, caused no quenching of fluorescence.

The effect of acetylsalicylic acid, ibufenac, indomethacin, oxyphenbutazone and phenylbutazone on the binding of 11-hydroxysteroids to proteins in the plasma of rheumatoid arthritic patients is recorded in Table 1. Only acetylsalicylic acid produced an increase in the proportion of unbound 11-hydroxysteroids. This displacement of 11-hydroxysteroids was complete after 30 min in contact with the acetylsalicylic acid, and remained constant over a period of 20 h. However, it was convenient to leave the acetylsalicylic acid in contact with the plasma overnight (16 h) and this practice was adopted throughout.

Displacement of 11-hydroxysteroids from their protein-binding sites by acetylsalicylic acid occurs to an equal extent in plasma obtained from normal volunteers and from patients with rheumatoid arthritis (Table 2). The proportion of unbound 11-hydroxysteroids in blood-bank plasma (four samples) was higher than in normal and rheumatoid arthritic human plasma (Table 2), possibly due to protein denaturation on storage. However, the displacement of 11-hydroxysteroids by acetylsalicylic acid in

Table 2. *Comparison of the effect of acetylsalicylic acid on the binding of* 11*-hydroxysteroids to proteins in normal, rheumatoid arthritic and blood-bank plasma*

| | No. | Total plasma 11-hydroxysteroids* μg/100 ml | Unbound 11-hydroxysteroids* μg/100 ml: Control | Unbound 11-hydroxysteroids* μg/100 ml: After acetylsalicylic acid | Percentage displacement |
|---|---|---|---|---|---|
| Normal plasma | 1 | 59·2 | 22·3 | 25·6 | 5·5 |
| | 2 | 65·2 | 17·7 | 22·1 | 6·8 |
| | 3 | 62·3 | 19·1 | 22·3 | 5·1 |
| | 4 | 53·7 | 16·3 | 20·2 | 7·2 |
| | 5 | 57·6 | 13·5 | 15·8 | 4·0 |
| | 6 | 60·4 | 14·6 | 20·6 | 9·9 |
| | 7 | 56·0 | 16·2 | 19·6 | 6·1 |
| | Mean | 59·2 | 17·1 | 20·9 | 6·37 |
| Rheumatoid arthritic plasma | 1 | 59·0 | 10·8 | 14·4 | 6·1 |
| | 2 | 59·8 | 14·1 | 18·1 | 6·7 |
| | 3 | 64·7 | 17·4 | 22·4 | 7·7 |
| | 4 | 63·0 | 17·9 | 21·7 | 6·0 |
| | 5 | 67·2 | 17·0 | 21·4 | 6·5 |
| | 6 | 60·7 | 20·4 | 23·1 | 4·5 |
| | 7 | 59·6 | 15·7 | 17·9 | 3·7 |
| | Mean | 62·0 | 16·2 | 19·9 | 5·89 |
| Blood bank plasma | 1 | 61·8 | 22·4 | 26·4 | 6·5 |
| | 2 | 57·4 | 21·7 | 24·7 | 5·2 |
| | 3 | 59·8 | 16·2 | 19·7 | 5·8 |
| | 4 | 64·0 | 23·0 | 26·8 | 6·0 |
| | Mean | 60·8 | 20·8 | 24·4 | 5·88 |

* Each result is the mean of two determinations.

Mean of 18 duplicate total plasma 11-hydroxysteroid concentration = 60·6 μg/100 ml.

Mean of 18 duplicate determinations of 11-hydroxysteroids in the same plasmas after addition of acetylsalicylic acid (1 mg/ml) (not recorded) = 58·8 μg/100 ml.

these samples was similar to that produced in normal and rheumatoid arthritic plasma (Table 2). Blood-bank plasma was used, therefore, in some later experiments to avoid the withdrawal of the large quantities of blood which would otherwise have been required.

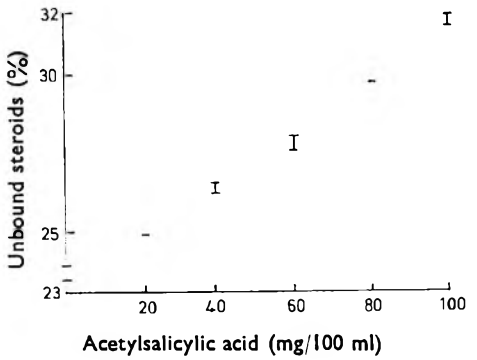


FIG. 1. Changes in the percentage of unbound 11-hydroxysteroids (shown as the range between duplicate determinations) in aliquots of blood-bank plasma at total 11-hydroxysteroid concentration of 63.2 μg/100 ml and varying concentrations of acetylsalicylic acid.

Fig. 1 shows that the displacement of 11-hydroxysteroids from the plasma protein-binding sites in blood-bank plasma is proportional to the concentration of acetylsalicylic acid. However, the curve indicates that the percentage of plasma total 11-hydroxysteroids which remains unbound at concentrations of acetylsalicylic acid normally found in the plasma of rheumatoid arthritic patients (20 mg/100 ml) is only some 1% greater than in untreated plasma. The clinical significance of this is, therefore, doubtful. Competition for binding sites, also is not great as it requires acetylsalicylic acid (1 mg/ml) to displace 37 ng/ml of 11-hydroxysteroids (Table 2). Further, since it is known that acetylsalicylic acid binds to human plasma albumin (Reynolds & Cluff, 1960), it seems likely that in binding, this drug causes displacement of 11-hydroxysteroids from albumin binding sites.

Since the drugs examined are all acidic and the pH of plasma is known to affect the binding ability of transcortin (the principal corticosteroid-binding globulin), with the optimum between pH 7 and 8 (Daughaday & Mariz, 1961), it was necessary also to

Table 3. *The effect of acetylsalicylic acid* (1 *mg/ml*) *on the displacement of* 11-*hydroxysteroids from plasma proteins at different total concentrations of* 11-*hydroxysteroids and calculated displacements from* (1) *all binding sites and* (2) *albumin-binding sites only*

| Added 11-hydroxy-steroids μg/100 ml | Total plasma 11-hydroxy-steroids† μg/100 ml (A) | Total plasma 11-hydroxy-steroids not bound to transcortin*† μg/100 ml (B) | Unbound 11-hydroxysteroids† | | Acetylsalicylic acid | | |
|---|---|---|---|---|---|---|---|
| | | | Control (C) | After acetyl-salicylic acid (D) | Displacement from all binding sites (D–C) μg/100 ml (E) | % Displacement from all binding sites (E/A × 100) | % Displacement from albumin-binding sites (E/B × 100) |
| 0 | 14·6 | 0‡ | 2·71 | 2·62 | −0·09 | −0·6 | — |
| 5 | 19·4 | 0‡ | 3·16 | 3·27 | 0·11 | 0·6 | — |
| 10 | 24·0 | 2‡ | 3·48 | 3·68 | 0·20 | 0·8 | 10 |
| 20 | 34·3 | 12·3 | 8·43 | 9·09 | 0·66 | 1·9 | 5·4 |
| 30 | 44·1 | 22·1 | 12·50 | 14·30 | 1·80 | 4·1 | 8·3 |
| 50 | 64·1 | 42·1 | 23·30 | 27·40 | 4·10 | 6·4 | 9·7 |

* Based on an assumed CBC of transcortin = 22·0 μg/100 ml.

† Each result is the mean of three determinations.

‡ Neglecting the unbound concentration.

consider the effect of the drugs on the pH of plasma, and hence on the binding of 11-hydroxysteroids. The pH values of blood-bank and rheumatoid arthritic plasmas, with raised levels of 11-hydroxysteroids, and containing acetylsalicylic acid, ibufenac, indomethacin, oxyphenbutazone and phenylbutazone are recorded in Table 4.

Blood-bank plasma has a lower pH (6·9) than the physiological pH (7·4) because of added sodium acid citrate. Also, fresh rheumatoid arthritic plasma, containing heparin anticoagulant, loses carbon dioxide on exposure to air; its pH is, therefore, slightly higher than physiological pH. Only acetylsalicylic acid had any appreciable effect on the pH of either plasma. Adjustment of plasma pH with hydrochloric acid (in place of acetylsalicylic acid) to the same pH as that obtained with acetylsalicylic acid did not alter the concentration of unbound 11-hydroxysteroids, indicating that their displacement from their protein-binding sites by the drug is not because of the accompanying fall in pH.

Table 4. *Effect of acetylsalicylic acid, indomethacin, ibufenac, oxyphenbutazone and phenylbutazone on the pH of plasma from a patient with rheumatoid arthritis and from blood-bank plasma*

| Source of plasma | Plasma aliquot number | Added drug | pH |
|---|---|---|---|
| Rheumatoid arthritic patient | 1 | None (Control) | 7·8 |
| | 2 | Acetylsalicylic acid (1 mg/ml) | 7·3 |
| | 3 | Ibufenac (0·1 mg/ml) | 7·7 |
| | 4 | Indomethacin (0·02 mg/ml) | 7·8 |
| | 5 | Oxyphenbutazone (0·2 mg/ml) | 7·8 |
| | 6 | Phenylbutazone (0·2 mg/ml) | 7·8 |
| Blood bank | 1 | None (Control) | 6·9 |
| | 2 | Acetylsalicylic acid (1 mg/ml) | 6·5 |
| | 3 | Ibufenac (0·1 mg/ml) | 6·8 |
| | 4 | Indomethacin (0·02 mg/ml) | 6·9 |
| | 5 | Oxyphenbutazone (0·2 mg/ml) | 6·9 |
| | 6 | Phenylbutazone (0·2 mg:ml) | 6·9 |

Displacement of 11-hydroxysteroids by acetylsalicylic acid (100 mg/100 ml) in blood-bank plasma (Table 3) was appreciable only at the higher 11-hydroxysteroid concentrations, apparently when the CBC of transcortin was exceeded; thereafter it increased with increase in total steroid concentration. The displacement from albumin-binding has been calculated (Table 3), assuming the CBC attributable to transcortin in blood-bank plasma to be 22 $\mu$g/100 ml (a low value, chosen since the percentage of unbound 11-hydroxysteroids in blood-bank plasma is apparently higher than in normal plasma) (Table 2). Evidence that displacements at these higher 11-hydroxysteroid concentrations only involve albumin-binding sites was obtained from parallel experiments with 4% human serum albumin containing hydrocortisone (25 $\mu$g/100 ml) with and without added acetylsalicylic acid (100 mg/100 ml). Ultrafiltrates showed an increase in unbound hydrocortisone levels to 76·4% from 65·2% in the control ultrafiltrate, a displacement of 11·2%, which compares favourably with the calculated displacements in Table 2.

That the displacement is mainly from albumin-binding sites is further confirmed in Table 5 which shows the effect of acetylsalicylic acid on the binding of 11-hydroxysteroids on the plasma from two rheumatoid arthritic patients, in whom the 11-hydroxysteroid levels have been artificially raised by injections of depot tetracosactide

(Synacthen). Seal & Doe (1962) have concluded that transcortin-binding sites are quickly saturated after the administration of ACTH, and that the balance of the 11-hydroxysteroids are either unbound or albumin bound. The results in Table 5 show that aspirin produces displacements of bound 11-hydroxysteroids to similar extents to those observed in the experiments with blood-bank plasma and with human serum albumin solutions.

Table 5. *Effect of acetylsalicylic acid on the protein-binding of 11-hydroxysteroids in the plasma from two rheumatoid arthritic patients, 4 h after an intramuscular injection of depot synacthen and from two normal women in late pregnancy*

| Source of plasma | No. | Total plasma 11-hydroxysteroids* µg/100 ml (A) | Unbound 11-hydroxysteroids* µg/100 ml | | Acetysalicylic acid | |
|---|---|---|---|---|---|---|
| | | | Control | After acetylsalicylic acid | Displacement µg/100 ml (B) | % Displacement $\frac{B \times 100}{A}$ |
| Rheumatoid arthritic patients, 4 h after an intramuscular injection of Depot Synacthen | 1 | 81·4 | 25·5 | 30·6 | 5·1 | 6·3 |
| | 2 | 40·0 | 7·1 | 8·5 | 1·4 | 3·5 |
| Normal women in late pregnancy | 1 | 40·2 | 2·77 | 2·93 | 0·16 | 0·4 |
| | 2 | 47·4 | 2·09 | 2·37 | 0·28 | 0·6 |

* Each result is the mean of two determinations.

Endogenous levels of 11-hydroxysteroids and apparently transcortin (or a similar protein) are increased in the plasma of women in the third trimester of pregnancy. Consequently the CBC for 11-hydroxysteroids is also raised. As expected, therefore, displacements (Table 5) of 11-hydroxysteroids by aspirin in the plasma of two women in late pregnancy were small (0·6 and 0·4%) compared with those observed in rheumatoid arthritic patients after injection of tetracosactide (6·3 and 3·5%), although the total levels of 11-hydroxysteroids were comparable.

Phenylbutazone (Burns Rose & others, 1953), indomethacin (Hucker, Zaccher & others, 1966), and ibufenac (Adams, S. S. & Cliff, E. E., personal communication) also bind strongly to plasma proteins. They are similar to many other non-steroidal, acidic anti-inflammatory drugs in that they inhibit heat coagulation of serum albumin and of whole serum as a result of this interaction (Mizushima, 1966; Mizushima & Kobayashi, 1968). In contrast to acetylsalicylic acid, however, the interaction of ibufenac, indomethacin and phenylbutazone with plasma proteins does not interfere with the protein-binding of 11-hydroxysteroids (Table 1). These results are in complete agreement with those obtained for indomethacin by Dr. R. H. Silber (personal communication) employing equilibrium dialysis at 5°.

Contrary to expectation, oxyphenbutazone caused a significant increase in the 11-hydroxysteroids bound to plasma proteins (Table 1). Many examples of one drug displacing another drug or hormone from its protein-binding sites are known (Brodie, 1965), but this, to our knowledge, is the first report of a drug decreasing the unbound concentration of a hormone. The phenomenon is under further investigation.

We conclude that the results of the present work confirm those of the *in vivo* experiments (Stenlake & others, 1968), and lead to the opinion that non-steroidal anti-inflammatory drugs do not exert their effect by causing an increase in the proportion of unbound 11-hydroxysteroids in human plasma.

*Acknowledgements*

The authors wish to thank Mr. G. Milne, Deputy Director, West of Scotland Blood Transfusion Service and Dr. MacAndrew, Royal Infirmary, Glasgow, for a generous supply of blood-bank plasma and to the manufacturers for supplying anti-rheumatic drugs. The work was carried out during the tenure by one of us (AGD) of the Jacob Bell Memorial Scholarship and F. C. J. Bird Award from the Pharmaceutical Society of Great Britain, and was supported by a grant from the Nuffield Foundation.

## REFERENCES

BRODIE, B. B. (1965). *Proc. Roy. Soc. Med.*, **58**, 946–955.

BURNS, J. J., ROSE, R. K., CHENKIN, T., GOLDMAN, A., SCHULERT, A. & BRODIE, B. B. (1953). *J. Pharmac. exp. Ther.*, **109**, 346–357.

BURNS, J. J., ROSE, R. K., GOODWIN, S., REICHENTHAL, J., HORNING, E. C. & BRODIE, B. B. (1955). *Ibid*, **113**, 481–489.

DAUGHADAY, W. H. & MARIZ, I. K. (1961). *Metabolism*, **10**, 936–950.

DE MOOR, P., HEIRWEGH, K., HEREMANS, S. F. & DECLERCK-RASKIN, M. (1962). *J. clin. Invest.*, **41**, 816–827.

HOLT, L. P. J. & HAWKINS, C. F. (1965). *Br. med. J.*, **1**, 1354–1356.

HUCKER, H. B., ZACCHER, A. G., COX, S. V. BRODIE, D. A. & CANTWELL, N. H. R. (1966). *J. Pharmac. exp. Ther.*, **153**, 237–249.

MATTINGLY, D. (1962). *J. clin. Path.*, **15**, 374–379.

MIZUSHIMA, Y. (1966). *Lancet*, **2**, 433.

MIZUSHIMA, Y. KOBAYASHI, M. (1968). *J. Pharm. Pharmac.*, **20**, 169–173.

REYNOLDS, R. C. & CLUFF, L. E. (1960). *Bull. Johns Hopkins Hosp.*, **107**, 278–290.

SEAL, U. S. & DOE, R. P. (1962). *J. biol. Chem.*, **237**, 3136–3140.

SMITH, P. K., GLEASON, H. L., STOLL, C. G. & OGORZALEX, S. (1946). *J. Pharmac. exp. Ther.*, **87**, 237–255.

STENLAKE, J. B., DAVIDSON, A. G., JASANI, M. K. & WILLIAMS, W. D. (1968). *J. Pharm. Pharmac.*, **20**, *Suppl.*, 248S–253S.

TORIBARA, T. Y. (1953). *Analyt. Chem.*, **25**, 1286.

RECHENBERG, H. K. v. & HERRMANN, B. (1961). *Schweiz. med. Wschr.*, **91**, 403–405.

YU, T. F., BURNS, J. J., PATON, B. C., GUTMAN, A. B. & BRODIE, B. B. (1958). *J. Pharmac. exp. Ther.*, **123**, 63–69.

*J. Pharm. Pharmac.*, 1969, **21**, 460–464 *Received April* 23, 1969

# Central and peripheral monoaminergic membrane-pump blockade by some addictive analgesics and antihistamines

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The ability of various analgesics and antihistamines to block the amine-uptake mechanism (the so-named membrane pump) of central and peripheral monoamine-storing neurons was investigated in mice. Activities indicating such blockade were observed within both groups but did not seem to correlate with analgesic or antihistaminic activity. The antihistamine chlorpheniramine proved remarkably potent on central 5-hydroxytryptamine neurons.

Previously we have investigated the membrane-pump blocking action of some di- and tricyclic thymoleptic drugs on central and peripheral noradrenaline and on central 5-hydroxytryptamine (5-HT) neurons (Carlsson, Corrodi & others, 1969a, b; Carlsson, Fuxe & others, 1969; Carlsson, Jonason & others, 1969). The degree of blockade differed in the three types of neuron depending on the chemical structure and physical properties of the agents investigated. Moreover, it was observed that agents possessing strong membrane-pump blocking activity on 5-HT neurons also potentiated the actions of 5-hydroxytryptophan and the monoamine oxidase inhibitor nialamide.

In man also, the combined treatment with monoamine oxidase inhibitors and tricyclic thymoleptics may give rise to strong, even fatal reactions. Similar reactions have been observed after monoamine oxidase inhibitors and pethidine. In rabbits Nymark & Møller-Nielsen (1963) made similar observations. In animals pretreated with a monoamine oxidase inhibitor they found that amitriptyline caused hyperthermia and various signs of excitation. Pethidine had similar actions following monoamine oxidase inhibition. We have confirmed the last-mentioned observation in rabbits and mice. The question then arose whether pethidine, like the tricyclic thymoleptics, was capable of blocking the membrane pumps of monoamine-carrying neurons. Attempts to answer this question led to the investigations described below.

## EXPERIMENTAL AND RESULTS

Several drugs were examined for nialamide and 5-hydroxytryptophan potentiation (Table 1). As mentioned, pethidine (50 mg/kg, i.p.) caused potentiation of both these agents, whereas methadone and morphine appeared to be inactive in this respect.

Membrane pump blockade was studied by utilizing the principle of endogenous 5-HT and noradrenaline displacement by the amines H 75/12 (Carlsson & others, 1969b) and H 77/77, respectively (Carlsson & others, 1969a). Pethidine (50 mg/kg, i.p.) inhibited 5-HT displacement by H 75/12 by about 50% (Table 2). Of other analgesics, methadone appeared to possess some activity in a dose of 12·5 mg/kg (higher doses could not be studied because of toxicity), whereas morphine, pentazocine, and nalorphine

Table 1. *Potentiation of nialamide and 5-hydroxytryptophan syndromes by analgesics and antihistamines.* Groups of 5 mice were treated with nialamide (100 mg/kg i.p.) 1 h before or 5-HTP (300 mg/kg, i.p.) 30 min after the test drug. The test drugs were given i.p. in doses indicated in brackets. In the doses employed neither nialamide nor 5-HTP caused any marked behavioural changes. When potentiation occurred, the gross symptoms induced by 5-HTP were extension and abduction of hind limbs, lordosis, tremors, head movements and excitation; the syndrome induced by nialamide was similar.

| | Nialamide potentiation | | 5-hydroxytryptophan potentiation | |
|---|---|---|---|---|
| Pethidine HCl | Yes | (50) | Yes | (50) |
| Methadone HCl | No | (12·5) | No | (12·5) |
| Morphine HCl | No | (50, 25) | No | (50) |
| Chlorpheniramine maleate | Yes | (50, 12·5) | Yes | (25) |
| Chlophedianol HCl | No | (50, 12·5) | Yes | (25) |
| Recipavrin HCl[1] | Yes | (50, 12·5) | Yes | (50) |
| Terodiline HCl[2] | Doubtful | (50, 12·5) | Doubtful | (25) |
| Diphenhydramine HCl | Yes | (50,[3] 12·5) | Yes | (25) |
| Chlorphenoxamine HCl | No | (50, 12·5) | Yes | (25) |
| Tripelennamine HCl | Yes | (50, 25, 12·5) | Yes | (25) |
| Promethazine HCl | No | (50, 12·5) | Doubtful | (25) |
| Phenindamine tartrate | Doubtful[4] | (50, 12·5) | Doubtful[4] | (25) |
| Cyproheptadine HCl | No | (50,[5] 12·5) | No | (25) |

[1] 4,4-diphenyl-2-dimethylaminobutane hydrochloride.
[2] *N*-Butyl-1-methyl-3,3-diphenylpropylamine hydrochloride
[3] Four out of 5 animals died within 60 min.
[4] Difficult to judge because of the stimulating effect of the drug itself.
[5] All animals died within 80 min.

had no significant activity. The structure of methadone is similar to that of certain antihistamines, which like the thymoleptics possess a dimethylaminopropyl side-chain. Moreover, certain antihistamines have been reported to block the membrane pump of peripheral adrenergic neurons, which would explain their noradrenaline-potentiating activity (Isaac & Goth, 1965, 1967), and to antagonize reserpine and tetrabenazine in various animal tests for thymoleptic activity (Garattini & Jori, 1967; Barnett, Taber & Roth, 1969).

We therefore tested several antihistamines for 5-hydroxytryptophan and nialamide potentiation (Table 1); some were active, others inactive. In general there seemed to be a good agreement between nialamide and 5-hydroxytryptophan potentiation. Only in two cases out of 13 was clear-cut potentiation observed with one of the agents, but not with the other. In both cases the negative result was obtained with nialamide, suggesting that the potentiation "threshold" is higher for this drug than for 5-hydroxytryptophan under the present conditions. When considered together with our earlier observations (Carlsson, Jonason & others, 1969), the data indicate a close association between nialamide and 5-hydroxytryptophan potentiation, suggesting that 5-HT is involved in both cases. The activities also correlated fairly well with those observed by the above-mentioned workers for blockade of peripheral adrenergic membrane pumps, noradrenaline potentiation and reserpine: tetrabenazine antagonism. Five antihistamines of varying chemical structure were examined for their ability to block H75/12-induced 5-HT displacement (Table 2). The two agents having a dimethylaminopropyl side-chain, i.e. chlorpheniramine and Recipavrin (used in Sweden as a

Table 2. *Effects of various drugs and* 4-*methyl-a-ethyl*-m-*tyramine* (*H* 75/12), *given alone or in combination, on the brain-5-hydroxytryptamine level in mice*

| Treatment | First dose (mg/kg) | Brain 5-HT (μg/g) Drug alone | Drug + H75/12 | Inhibition (%) | Treatment | First dose (mg/kg) | Brain 5-HT (μg/g) Drug alone | Drug + H75/12 | Inhibition (%) |
|---|---|---|---|---|---|---|---|---|---|
| No drug | | 0·54 (7) ±0·018 | 0·23 (15) ±0·011 | | No drug | | 0·54 (7) ±0·018 | 0·23 (15) ±0·011 | |
| Pethidine HCl | 50 | 0·55 (3) ±0·056 | 0·41 (3) ±0·014 | 56 | Recipavrin HCl | 25 | 0·62 (2) ±0·100 | 0·41 (2) ±0·035 | 46 |
| | 25 | 0·59 (2) ±0·015 | 0·29 (2) ±0·020 | 17 | | 12·5 | 0·53 (2) ±0·020 | 0·38 (2) ±0·055 | 50 |
| Methadone HCl | 12·5 | 0·59 (3) ±0·012 | 0·31 (3) ±0·032 | 22 | | 6·25 | 0·61 (1) | 0·34 (1) | 29 |
| Morphine HCl | 50 | 0·54 (4) ±0·038 | 0·27 (4) ±0·036 | 13 | Diphenhydramine HCl | 25 | 0·56 (2) ±0·025 | 0·44 (2) ±0·055 | 64 |
| | | | | | | 12·5 | 0·56 (2) ±0·095 | 0·29 (2) ±0·025 | 18 |
| Pentazocine | 25 | 0·67 (1) | 0·24 (1) | 2 | Tripelennamine HCl | 25 | 0·53 (2) ±0·025 | 0·33 (2) ±0·015 | 33 |
| | 12·5 | 0·55 (1) | 0·27 (1) | 13 | | 12·5 | 0·58 (2) ±0·015 | 0·27 (2) ±0·040 | 11 |
| Nalorphine HCl | 25 | 0·57 (2) ±0·045 | 0·24 (2) ±0·010 | 3 | | 6·25 | 0·49 (1) | 0·18 (1) | 0 |
| Chlorpheniramine maleate | 25 | 0·57 (3) ±0·035 | 0·52 (2) ±0·040 | 85 | Phenindamine tartrate | 25 | 0·59 (2) | 0·29 (1) | 17 |
| | 12·5 | 0·53 (3) ±0·030 | 0·42 (3) ±0·021 | 63 | | 12·5 | 0·60 (2) | 0·27 (1) | 11 |
| | 6·25 | 0·63 (1) | 0·41 (1) | 45 | | | | | |

Shown are the means ± s.e. Figures in brackets indicate number of experimental groups, each comprising 5 animals.

Table 3. *Effects of various drugs and* 4,*a-dimethyl*-m-*tyramine* (*H* 77/77), *given alone or in combination, on noradrenaline in brain and heart*

| Treatment | First dose mg/kg | Brain noradrenaline μg/g Drug alone | Drug+ H77/77 | Heart noradrenaline μg/g Drug alone | Drug+ H77/77 | % Inhibition Brain | Heart |
|---|---|---|---|---|---|---|---|
| None | | 0·40 (4) ±0·030 | 0·18 (6) ±0·013 | 0·60 (4) ±0·029 | 0·11 (6) ±0·022 | | |
| Pethidine HCl | 50 | 0·38 (2) ±0·000 | 0·24 (2) ±0·055 | 0·54 (2) ±0·120 | 0·17 (3) ±0·023 | 30 | 14 |
| Methadone HCl | 12·5 | 0·35 (1) | 0·16 (1) | 0·67 (1) | 0·13 (1) | 0 | 4 |
| Morphine HCl | 50 | 0·40 (1) | 0·17 (1) | 0·61 (1) | 0·11 (1) | 0 | 0 |
| Chlorpheniramine | 25 | 0·42 (3) ±0·047 | 0·32 (3) ±0·046 | 0·71 (3) ±0·087 | 0·53 (3) ±0·054 | 58 | 70 |
| Maleate | 12·5 | 0·43 (3) ±0·055 | 0·27 (3) ±0·045 | 0·86 (3) ±0·165 | 0·61 (3) ±0·141 | 36 | 67 |
| Recipavrin HCl | 25 | 0·52 (1) | 0·24 (1) | 0·79 (1) | 0·54 (1) | 18 | 63 |
| | 12·5 | 0·48 (2) ±0·030 | 0·24 (2) ±0·015 | 0·90 (2) ±0·045 | 0·39 (2) ±0·150 | 20 | 35 |
| | 6·25 | 0·47 (1) | 0·20 (1) | 1·16 (1) | 0·31 (1) | 7 | 19 |
| Diphenhydramine HCl | 12·5 | 0·36 (1) | 0·16 (1) | 0·50 (1) | 0·25 (1) | 0 | 36 |
| Tripelennamine HCl | 25 | 0·33 (2) ±0·010 | 0·18 (2) ±0·010 | 0·59 (2) ±0·045 | 0·30 (2) ±0·010 | 0 | 40 |
| | 12·5 | 0·40 (1) | 0·17 (1) | — | 0·16 (1) | 0 | — |
| | 6·25 | 0·36 (1) | 0·17 (1) | 0·75 (1) | 0·20 (1) | 0 | 14 |
| Phenindamine tartrate | 25 | 0·28 (2) ±0·025 | 0·24 (2) ±0·015 | 0·71 ±0·155 | 0·49 ±0·145 | 60 | 63 |
| | 12·5 | 0·32 (1) | 0·23 (1) | 0·66 (1) | 0·34 (1) | 36 | 42 |

Shown are the means ± s.e. Figures in brackets indicate number of experimental groups, each comprising 6 mice.

spasmolytic), proved most active. Tripelennamine, with an ethylenediamine side-chain, had some activity, and this was also true of diphenhydramine, an aminoalkyl-ether. Phenindamine and promethazine (Carlsson & others, 1969b) had little or no activity.

In general there appeared to be a good agreement between nialamide: 5-hydroxy-tryptophan potentiation and blockade of H 75/12-induced 5-HT displacement (*cf.* Tables 1 and 2), supporting our previous observations (Carlsson, Jonason & others, 1969). Both phenomena may therefore well be due to one and the same basic action, that is, blockade of the membrane pump of 5-HT neurons.

Three analgesics and five antihistamines were examined for their ability to block H 77/77-induced noradrenaline depletion (Table 3). Chlorpheniramine proved active on central noradrenaline but was probably less potent than on 5-HT. Recipavrin was less active. Tripelennamine and diphenhydramine (one experiment only) appeared to be inactive on central noradrenaline. On peripheral noradrenaline (heart) all four compounds appeared to be active. The findings agree with our earlier observations that membrane pump blockade by various agents is generally more pronounced in peripheral than in central noradrenaline neurons. Phenindamine, an antihistamine with central stimulant properties in animals and man, was peculiar in reducing brain noradrenaline levels by itself. It also seemed rather efficient in blocking H 77/77-induced noradrenaline release centrally as well as peripherally. Further analysis of these observations may throw some light on the central stimulation caused by this agent. The analgesics examined had little or no activity.

In agreement with our earlier data on di- and tricyclic thymoleptics, the types of drugs investigated did not seem to influence H 77/77-induced dopamine displacement, thus further supporting the view that the structural requirements for blockade of the membrane pumps of noradrenaline and dopamine neurons are different.

## DISCUSSION

It would thus appear that certain addictive analgesics and antihistamines possess some biochemical and pharmacological properties similar to those of the thymoleptic drugs. The question arises whether the agents in question do indeed have antidepressant activity in man. It is interesting to note that opium was used as an antidepressant agent before the advent of the modern thymoleptics. However, in the present study morphine showed little or no activity. It might prove interesting to investigate the other components of opium. In any event there does not seem to be any clearcut correlation between analgesic effect and the biochemical activities observed in this investigation. As to the antihistamines, it appears that only diphenhydramine has been examined for antidepressant activity in man. The result was considered negative (Hankoff, Grundlach & others, 1964) but the conclusion has been disputed (Barnett & others, 1969). Chlorpheniramine proved considerably more potent than diphenhydramine in the present study. In fact it compares favourably with imipramine and amitriptyline with respect to actions on both 5-HT and noradrenaline neurons. It appears worth while to study the possible antidepressant properties of this and related agents in man.

*Acknowledgements*

This work was supported by the Swedish Medical Research Council (B69-14X-155-05A and B69-14X-2157-03A). For generous gifts of drugs we are indebted to the

following companies: Asta-Werke (Systral, chlorphenoxamine), Bayer Ltd. (Refugal, chlophedianol), CIBA Ltd. (Pyribenzamin, tripelennamine), Hoffmann-La Roche & Co. Ltd. (Thephorin, phenindamine), Hässle Ltd., Dr. H. Corrodi (H 75/12 and H 77/77), Merck Sharp and Dohme Ltd. (Cyproheptadine), Parke-Davis Ltd. (Benadryl, diphenhydramine, Pfizer Ltd. (Nialamide), Pharmacia Ltd. (Allergisan, chlorpheniramine), Recip Ltd. (Bicor, terodiline, Lergigan, promethazine and Recipavrin) and Winthrop Ltd. (Fortalgesic, pentazocine). The skilful technical assistance of Mrs. Ingrid Bergh and Miss Birgitta Schultz is gratefully acknowledged.

## REFERENCES

BARNETT, A., TABER, R. I. & ROTH, F. E. (1969). *Int. J. Neuropharmac.*, **8**, 73–79.

CARLSSON, A., CORRODI, H., FUXE, K. & HÖKFELT, T. (1969a). *Eur. J. Pharmac.*, **5**, 357–366.

CARLSSON, A., CORRODI, H., FUXE, K. & HÖKFELT, T. (1969b). *Ibid.*, **5**, 367–373.

CARLSSON, A., FUXE, K., HAMBERGER, B. & MALMFORS, T. (1969). *Br. J. Pharmac.*, in the press.

CARLSSON, A., JONASON, J., LINDQVIST, M. & FUXE, K. (1969). *Brain Res.*, **12**, 456–460.

GARATTINI, S. & JORI, A. (1967). *Antidepressant drugs.* Proceedings of the first international symposium organised by The Istituto di Ricerche Farmacologiche "Mario Negri", Milan, April 25–27, 1966. Eds: S. Garattini, M. N. G. Dukes. International Congress Series No. 122. p. 179–193. Amsterdam: Excerpta Medica Foundation.

HANKOFF, L. D., GRUNDLACH, R. H., PALEY, H. M. & RUDOLFER, L. (1964). *Dis. nerv. Syst.*, **25**, 547–553.

ISAAC, L. & GOTH, A. (1965). *Life Sci.*, **4**, 1899–1904.

ISAAC, L. & GOTH, A. (1967). *J. Pharmac. exp. Ther.*, **156**, 463–468.

NYMARK, M. & MØLLER NIELSEN, I. (1963). *Lancet*, **2**, 524–525.

*J. Pharm. Pharmac.*, 1969, **21**, 465

# LETTERS TO THE EDITOR

## Interaction between cerebral amines and 4-hydroxybutyrate in the induction of sleep

Little is known about the mechanism of action of short-chain fatty acids and their derivatives on sleep induction (Jouvet, 1967a). An action at the level of serotoninergic or noradrenergic reticular ascending systems is conceivable. Experiments concerning a possible conversion of 4-hydroxybutyrate to γ-aminobutyrate and vice versa (Della Pietra, Illiano & others, 1966; Mitoma & Neubauer, 1968; Roth & Giarman, 1969) and the effect of 4-hydroxybutyrate on cerebral glucose metabolism (Laborit, 1964; Godin, Mark & Mandel, 1968) have failed to answer this problem. The investigations of Gessa, Crabai & others (1966, 1968) have pointed out that intraperitoneal 4-hydroxybutyrate produces a rise of cerebral dopamine in rats and rabbits. Whether this is a coincident or a causal finding is still debatable.

To test if dopamine influences the hypnotic action of 4-hydroxybutyrate we gave L-dopa (the natural precursor of this amine) intraperitoneally to mice. Since fully hypnotic doses of 4-hydroxybutyrate proved to be convulsive in some animals we injected a reduced amount of the drug. Thirty min later the animals were injected by the same route with 4-hydroxybutryate. Controls received dopa followed by saline. To exclude any aspecific synergism we gave mice, similarly pretreated with dopa, a dose of pentobarbitone. Sleep onset and duration were judged by the loss and the return of righting reflex. Results are summarized in Table 1.

It is evident that dopa strongly enhanced the hypnotic effect of 4-hydroxybutyrate, whereas its influence on the action of pentobarbitone was minimal and not significant.

These results are of some interest as the administration of L-dopa alone has been shown to produce an increase in waking (Jouvet, 1967b).

Table 1. *Effect of a load of* L-dopa *on sleep induced by 4-hydroxybutyrate (γ-OH) and pentobarbitone*

| No. of mice | Drug | Duration of sleep (±s.e.) (min) | % Asleep | Lag time (±s.e.) (min) |
|---|---|---|---|---|
| 9 | L-Dopa (50 mg/kg) .. .. | — | 0 | — |
| 20 | 4-Hydroxybutyrate (500 mg/kg) .. .. | 2 ± 0 | 10 | 8 ± 1 |
| 20 | L-Dopa (50 mg/kg) + γ-OH (500 mg/kg) | 27 ± 6* | 40 | 8 ± 2 |
| 25 | 4-Hydroxybutyrate (750 mg/kg) .. .. | 4 ± 1 | 56 | 6 ± 1 |
| 25 | L-Dopa (50 mg/kg) + γ-OH (750 mg/kg) .. .. | 34 ± 6* | 68 | 7 ± 1 |
| 12 | Pentobarbitone (38 mg/kg) | 30 ± 7 | 58 | |
| 12 | L-Dopa (50 mg/kg) + pentobarbitone (38 mg/kg) | 40 ± 10 | 58 | |
| 12 | Pentobarbitone (30 mg/kg) | — | 0 | |
| 12 | L-Dopa (50 mg/kg) + pentobarbitone (30 mg/kg) | — | 0 | |

* $P > 0{\cdot}01$, *t*-test

The purpose of these experiments was to check the hypothesis that a direct interaction between fatty acid molecule and chemical transmitters of noradrenergic systems can account for sleep induction (Rizzoli & Galzigna, 1969). The present experiments do not exclude such an hypothesis.

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REFERENCES

DELLA PIETRA, G., ILLIANO, G., CAFANO, V. & RAVA, R. (1966). *Nature, Lond.*, **210**, 733–734.

GESSA, G. L., VARGIU, L., CRABAI, F., BOERO, G. C., CABONI, F. & CAMBA, R. (1966). *Life Sci.*, **5**, 1921–1930.

GESSA, G. L., SPANO, P. F., VARGIU, L., CRABAI, F., TAGLIAMONTE, A. & MAMELI, L. (1968). *Ibid.*, **7**, 289–298.

GESSA, G. L., CRABAI, F., VARGIU, L. & SPANO, P. F. (1968). *J. Neurochem.*, **15**, 377–381.

GODIN, Y., MARK, J. & MANDEL, P. (1968). *Ibid.*, **15**, 1085–1091.

JOUVET, M. (1967a). In *The Neurosciences: a study program*, Editors: Quarton, T. C., Melnechuck, T., & Schmitt, F. O. pp. 529–545. The Rockefeller University Press.

JOUVET, M. (1967b). In *Sleep and altered states of consciousness* Editors: Kety, S. S., Evarts, E. V. & Williams, H. L., Baltimore: William and Wilkins.

LABORIT, H. (1964). *Int. J. Neuropharmac.*, **3**, 433–451.

MITOMA, Ch. & NEUBAUER, S. E. (1968). *Experientia*, **24**, 12–13.

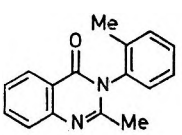
RIZZOLI, A. A. & GALZIGNA, L. (1969). II° Int. Meeting Neurochemical Society.

ROTH, R. H. & GIARMAN, N. J. (1969). *Biochem. Pharmac.*, **18**, 247–250.

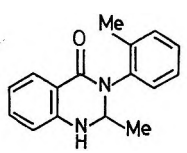
# Fluorimetric assay of methaqualone in plasma by reduction to 1,2,3,4-tetrahydro-2-methyl-4-oxo-3-*o*-tolylquinazoline

There is a need for a method of assay of therapeutic plasma levels of the hypnotic drug methaqualone (I). Several indirect methods of estimating the drug necessitate acid or alkaline hydrolysis to diazotizable amines (Maggiorelli & Gangemi, 1964; Nakano, 1964), but these lack specificity. Ultraviolet spectrophotometry offers more attractive quantitative procedures (Akagi, Oketani & Takada, 1964; Lawson & Brown, 1967), but the sensitivity is restricted by interference from biological blanks.

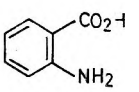
The structural resemblance between the dihydro-derivative of methaqualone (II) and anthranilic acid (III), an efficient fluorophor, suggested that a fluorimetric assay might be developed for methaqualone if a suitable reducing agent could be found. Okumura, Oine & others (1968) reduced some quinazolinone hydrochlorides with sodium borohydride, but the free bases were resistant to this reagent except under conditions which led to ring scission. We have found, however, that lithium borohydride is effective in reducing both free methaqualone and its hydrochloride to the tetrahydroquinazolinone




I




II




III

(II) and that this is stable in the presence of a large excess of reagent. Since (II) does show intense ultraviolet fluorescence, its preparation has been made the basis of a highly sensitive and specific assay for methaqualone in plasma.

*Preparation and characterization of* 1,2,3,4-*tetrahydro*-2-*methyl*-4-*oxo*-3-o-*tolyl-quinazoline* (*II*). An ice-cooled, stirred suspension of methaqualone hydrochloride (500 mg) in tetrahydrofuran (10 ml; freshly redistilled from sodium) was treated dropwise during 5 min with a suspension of lithium borohydride (60 mg; Fluka) in tetrahydrofuran (10 ml.) Completion of the reaction was indicated by dissolution of the hydrochloride, and development of a yellow colour. After stirring (15 min), water (2 ml) was added, and the solution evaporated to dryness. The residue was triturated with water (2 × 10 ml), desiccated, and recrystallized from ethyl acetate to give the tetrahydroquinazolinone (250 mg), as rhombs m.p. 197–8°. The findings in Table 1 confirm the structure assigned to the quinazolinone and show its freedom from methaqualone.

Table 1. *Comparison of some chromatographic and spectral properties of methaqualone and* 1,2,3,4-*tetrahydro*-2-*methyl*-4-*oxo*-3-o-*tolylquinazoline*

| Property | Methaqualone | Tetrahydroquinazolinone |
|---|---|---|
| Rf, thin-layer chromatography on MN-polyamide; aqueous ethanol | 0·72 | 0·51 |
| Rf, thin-layer chromatography on silica; chloroform–acetone–acetic acid | 0·62 | 0·69 |
| Retention time (min); gas chromatography on SE 30 at 187° | 10·5 | 20 |
| Infrared absorption maxima ($\mu$m); ATR spectra on KRS plates— | | |
| C=O | 6·0, strong | 6·2, strong |
| C=N | 6·3, strong | — absent |
| N—H | — absent | 3·1, medium |
| Ultraviolet absorption maxima in ethanol— $\lambda_{max}$nm (log E) | 225 (4·6) | 223 (4·5) |
| | 266 (4·0) | — |
| | 295 (3·5) | — |
| | 305 (3·6) | — |
| | 316 (3·4) | — |
| | — | 342 (3·5) |
| Fluorescence spectra in methanol; activation/emission maxima (nm) | none | 345/450 |

Fluorescence spectra of the tetrahydroquinazolinone were measured with an Aminco-Bowman spectrophotofluorimeter (xenon arc lamp with off-axis ellipsoidal mirror; 1 P 21 photomultiplier). The fluorescence characteristics in aqueous buffers were similar to those in organic solvents, and showed little change between pH 3–10.

At the activation and emission optima, linear log-log working curves for the various slit arrangements are obtained over a wide range of concentrations (0·01 to 100 $\mu$g/ml). The limit of detection of the pure compound is about 2 ng in 0·2 ml. The activation optimum at 345 nm, corresponding to the minor absorption peak in the ultraviolet spectrum at this wavelength, is shifted to 365 nm if a xenon/mercury arc is used as the exciting source, because of increased light output by the lamp at this wavelength. Under these conditions, the narrower slit arrangements can be employed for a given instrumental sensitivity, and this facilitates the use of microcuvettes. Efficient activation at the mercury emission line would also provide an advantage when working with filter fluorimeters not equipped with a white light source.

*Micro assay of methaqualone in plasma.* Heparinized plasma (50 $\mu$l) is extracted with Analar ethyl acetate (2 ml; redistilled) on a mechanical shaker for 10 min. After brief centrifugation, an aliquot (1·8 ml) of the supernatant is extracted with 0·05 M sodium hydroxide (50 $\mu$l), and separated similarly. The organic phase (1·6 ml) is evaporated to dryness, and the residue treated with a solution of lithium borohydride in anhydrous ether (2 ml of the supernatant from a freshly prepared suspension of nominal concentration 2 mg/ml). After 20 min, a small precipitate is removed by centrifugation and the solution evaporated to dryness. The residue is treated with Analar methanol (1 ml) to hydrolyse the reduction complex and unchanged reagent, and the solution is left for a few minutes until effervescence ceases. An aliquot is then taken for measurement of the fluorescent intensity relative to known standards and to a reagent blank. Suitable concentrations of the tetrahydroquinazolinone itself may be used for day-to-day standardization of the fluorimeter; alternatively, known amounts of methaqualone may be added to normal plasma and carried through the reaction sequence. Recoveries in the range 80 to 90% were readily obtained in this way with 0·1 to 6 $\mu$g quantities of methaqualone added to 50 $\mu$l samples of normal plasma, in spite of the fact that the drug is strongly protein bound.

By extracting methaqualone from plasma at physiological pH and washing the extract with alkali, a large measure of specificity can be achieved since many common drugs and their metabolites are acidic in character. Thus the barbiturates, salicylate and paracetamol do not interfere with the estimation. Interference might be expected from substances such as glutethimide, meprobamate or methyprylone, but these compounds have not been found to give rise to fluorescent derivatives under the conditions of the assay. Drugs which are themselves strongly fluorescent, and which would remain in the organic phase, such as quinidine or lysergic acid diethylamide might also interfere with the method, but this could be checked by measuring the fluorescence of the extract in a suitable solvent before carrying out the reduction.

This procedure can be used without modification for the assay of plasma methaqualone in poisoned patients, in whom levels almost invariably exceed 5 $\mu$g/ml (Matthew, Proudfoot & others, 1968). For the determination of therapeutic levels of the drug, 100 or 150 $\mu$l aliquots of plasma are taken, so as to give a final fluorescent intensity of at least twice that of the reagent blank. The blank itself is largely derived from the excess of lithium borohydride which, after methanolysis, shows weak fluorescence (activation/emission maxima, 310/375 nm).

In specimens from 15 patients on therapeutic regimes of Mandrax (Roussel), plasma levels of methaqualone approximately 12 h after ingestion were found to range from 0·9 to 2·2 $\mu$g/ml (mean and standard deviation, 1·5 $\pm$ 0·5 $\mu$g/ml).

We are grateful to Roussel Laboratories Ltd. for a gift of methaqualone.

This research program has been supported by a grant from the Scottish Hospital Endowments Research Trust.

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REFERENCES

AKAGI, M., OKETANI, Y. & TAKADA, M. (1963). *Chem. pharm. Bull., Tokyo*, **11**, 62–67.
LAWSON, A. A. H. & BROWN, S. S. (1967). *Scott. med. J.*, **12**, 63–68.
MAGGIORELLI, E. & GANGEMI, G. (1964). *Boll. chim.-farm.*, **103**, 748–752.
NAKANO, M. (1964). *Yakuzaigaku*, **24**, 49–52; *Chem. Abs.* 1964, **61**, 4155.
MATTHEW, H., PROUDFOOT, A. T., BROWN, S. S. & SMITH, A. C. A. (1968). *Br. med. J.*, **2**, 101–102.
OKUMURA, K., OINE, T., YAMADA, Y., HAYASHI, G. & NAKAMA, M. (1958). *J. medl Chem.*, **11**, 348–352.

# Acetylcholine content in the brain of rats treated with paraoxon and pyridinium-2-aldoxime methylchloride

Evidence indicating that pyridinium-2-aldoxime methylchloride (pralidoxime, P-2-AM) reactivates functional acetylcholinesterase in the brain of rats *in vivo* has been obtained by Milošević & Andjelković (1966) and Hobbiger & Vojvodić (1967). I have now investigated whether P-2-AM prevents the accumulation of acetylcholine in the brain of animals given a lethal dose of paraoxon.

Male albino rats, 150 to 200 g, were injected subcutaneously with 0·5 mg/kg of (*O*,*O*-diethyl-*O*-*p*-nitrophenyl) phosphate (paraoxon, E 600). Ten min later, 20 mg/kg of P-2-AM (chloride) was given intravenously and, 1 h after, the animals were decapitated. The brain, excluding the cerebellum was removed without delay and the total acetylcholine extracted with hydrochloric acid without the addition of physostigmine, by the method of Elliott, Swank & Henderson (1950).

The acetylcholine content of the extracts was estimated on the ileum of the guinea-pig in oxygenated Tyrode solution containing neostigmine, 5 μg/ml, and morphine, 10 mg/litre (Paton, 1957). Amounts of acetylcholine were assessed as chloride equivalents. In additional experiments, it was shown that the extracts did not contain the drugs used or substances other than acetylcholine in concentrations which affected the sensitivity of the assay preparation.

A lethal dose of 0·5 mg/kg of paraoxon increased the total amount of acetylcholine in the brain by approximately 80% (Table 1). Rats injected intravenously with P-2-AM survived, but the acetylcholine content in the brain of these animals was even greater than that in animals treated with paraoxon only.

Table 1. *Total acetylcholine content (mean ± s.e. μg/g tissue) in the brain of rats treated with paraoxon and P-2-AM*

| Substance (mg/kg) | No of rats | Time (h) | Acetylcholine | Change (%) |
|---|---|---|---|---|
| None | 15 | — | 2·9 ± 0·3 | — |
| Paraoxon (0·5) | 10 | 0·5* | 5·1 ± 0·4 | +75 |
| Paraoxon (0·5) + P-2-AM (20) | 10 | 1 | 5·8 ± 0·5 | +100 |
| | 10 | 2 | 7·5 ± 0·8 | +158 |
| | 8 | 4 | 4·3 ± 0·3 | +48 |
| | 8 | 6 | 3·9 ± 0·3 | +34 |
| P-2-AM (20) | 10 | 1 | 2·6 ± 0·3 | — |

* All animals died within 30 min of injecting paraoxon.

These findings indicate that neither the extent of P-2-AM-induced reactivation of phosphorylated functional acetylcholinesterase in the brain (Hobbiger & Vojvodić, 1967), nor redistribution of accumulating acetylcholine from the brain to the periphery containing active enzyme (Schaumann, 1960) can prevent the increase of brain acetylcholine induced by paraoxon. However, in spite of an abnormally high concentration of acetylcholine in the brain, the rats treated with P-2-AM exhibited no symptoms of central origin such as convulsions or tremor. The results are reminiscent of those of Brodeur & Dubois (1964) who found that in the presence of high concentrations of acetylcholine after inhibition of acetylcholinesterase, the cholinergic receptors may become less sensitive to acetylcholine.

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REFERENCES

BRODEUR, J. & DUBOIS, K. P. (1964). *Archs int. Pharmacodyn. Thér.*, **149**, 560–570.
ELLIOTT, K. A. C., SWANK, R. L. & HENDERSON, N. (1950). *Am. J. Physiol.*, **162**, 469–474.
HOBBIGER, F. & VOJVODIĆ, V. (1967). *Biochem. Pharmac.*, **16**, 455–462.
MILOSEVIĆ, M. P. & ANDJELKOVIC, D. (1966). *Nature, Lond.*, **210**, 206.
PATON, W. D. M. (1957). *Br. J. Pharmac. Chemother.*, **12**, 119–127.
SCHAUMANN, W. (1960). *Ibid.*, **15**, 432–435.

## Determination of dihydroxypropyltheophylline in plasma

There are numerous methods in the literature for the detection of dihydroxypropyltheophylline (diprophylline) in various solutions, but none are sensitive enough for its determination in plasma after therapeutic doses (Bukowska & Gierlowska, 1960, Ott & Wittman-Zinke, 1958; Raber, 1964).

The procedure of Schack & Waxler (1949) for theophylline has now been modified for the determination of diprophylline in plasma.

The procedure involves an extraction of the drug from plasma into chloroform–isopropanol (10:1) from which the drug is re-extracted with 20% v/v sulphuric acid. The absorbance of this solution is then read in a spectrophotometer. The absorbance of a standard solution of the drug in 20% v/v sulphuric acid is also measured. The peak absorbance is at 268 nm. The absorbance peak for theophylline in 0·1 N sodium hydroxide is at 277 nm.

*Method.* Plasma (2·0 ml) is extracted with chloroform (spectrograde)—isopropanol (nanograde) (10:1) (50 ml) by shaking vigorously for 10 min in a 120 ml separatory funnel. The solvent layer is then filtered through anhydrous sodium sulphate, the filter washed with fresh solvent (1 ml) and the filtrates combined. The plasma (aqueous) layer is then extracted a second time with another portion of chloroform-isopropanol (50 ml) for 10 min and the organic layer filtered to remove water as above and added to the previous 50 ml portion. The combined solvent layers (102 ml) are evaporated on a water bath to a final volume of 10 ml. This is placed in a 30 ml vial and extracted with 20% v/v sulphuric acid (reagent grade; 4·0 ml) by shaking vigorously for 10 min. The vial is then centrifuged to break the slight emulsion that forms. The sulphuric acid layer is pipetted into a cuvette and the absorbance read at 268 nm against a reagent blank consisting of plasma samples without diprophylline treated in the same way as the samples.

The percent recovery of diprophylline is 85–90% in the range of 10·0 to 50 0 μg/ml of plasma. The method is equally effective for human and rat plasma, is simple and fairly rapid and inexpensive.

The work was supported in part by Savage Laboratories, Inc., Bellaire, Texas.

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REFERENCES

BUKOWSKA, H. & GIERLOWSKA, J. (1960). *Chemia analit.* (Warsaw) 5, 309–315.
OTT, R. & WITTMAN-ZINKE, H. (1958). *Scientia Pharm.*, **26**, 217–224.
RABER, H. (1964). *Ibid.*, **32**, 122–127.
SCHACK, J. A. & WAXLER, S. H. (1949). *J. Pharmac. exp. Ther.*, **97**, 283–291.
TRUITT, E. B., CARR, C. J., BUBERT, H. M. & KRANTZ, J. C. (1947). *Ibid.*, **91**, 185–189.

# A technique for intrapleural cannulation in experimental animals

Quantitative determinations of the mechanical properties of the respiratory system often necessitate measurement of the pressure acting across the lungs, the so-called transpulmonary pressure. This pressure gradient can be determined by measuring the pressure outside the lungs but inside the chest wall (i.e., the intrapleural pressure) and subtracting the pressure at the opening to the airway. In man, intrapleural pressure changes can be estimated from the pressure in a small compliant balloon partially filled with air and inserted into the thoracic oesophagus. In anaesthetized laboratory animals it is convenient to measure intrapleural pressures directly. In practice, a cannula, which commonly consists of a hypodermic needle or similar device, is inserted through one of the intercostal spaces directly into the pleural cavity of the animal (Nadel & Widdicombe, 1962). Pressure changes within the intrapleural space are then transmitted via an air or liquid-filled system to a suitable pressure recording apparatus.

We encountered a number of problems with such a procedure for obtaining intrapleural pressures in anaesthetized dogs. For example, if the cannula was placed too near the myocardium, the intrapleural pressure waves had cyclic variations synchronous with the heart beat superimposed upon them. At times these superimpositions were large enough to make interpretation of respiratory events difficult and this in turn necessitated removal and relocation of the cannula. The physical nature of the hypodermic needle also presented problems. Unless held firmly in place by a cumbersome arrangement of clamps, the needle often moved during chest excursions and occasionally ceased transmitting pressures. In addition, small blood clots and other tissue debris occasionally became lodged in the tip of the needle, and so gradually dampened the pressure fluctuations. Further, post-mortem examination of animals often revealed a localized area of damaged lung tissue at the site of the cannulation procedure, presumably caused by continuous sliding of the involved lobe over the sharp point of the needle.

We now describe a technique similar to that of Amdur & Mead (1955) which we have used successfully to measure intrapleural pressure in anaesthetized dogs and which circumvents some of the aforementioned difficulties. The cannula which we use consists of a length of polyethylene tubing (PE 205), one end of which is connected to an appropriate pressure transducer. To minimize the likelihood of extraneous matter interfering with pressure transmission, we drill a number of small holes in the terminal inch of the cannula. Burrs around the drill holes are smoothed. The thoracic cavity is penetrated using a three to four inch length of twelve gauge, stainless steel needle stock, bevelled slightly at one end. Equal success in entering the pleural space has been achieved either by penetrating the chest wall at the level of the 5th or 6th intercostal space or by penetrating cephalad through the diaphragm. Using the former approach, the skin and intercostal muscles are punctured at a point along the upper surface of a rib to avoid damage to the intercostal blood vessels. The cannula, previously placed within the lumen of the steel tubing, is then slowly advanced into the thoracic cavity until pressure fluctuations in phase with respiration are observed. If excessive, cardiac-related superimpositions appear in the pressure record, the cannula is gently manoeuvered until a point is found at which the pressure trace is relatively smooth, and the steel tubing withdrawn. We find that by inserting the steel tubing at an angle perpendicular to the long axis of the animal and slightly lateral to the midline, the cannula can be guided into the thoracic cavity parallel to the inner aspect of the chest wall where cardiac effects are minimal.

The outside diameter of the cannula (0·082 inch) nearly coincides with the inside

diameter of the steel tubing (0·085 inch). These dimensions are selected to assure against the accidental creation of a large pneumothorax during cannulation. Indeed, we sometimes find it necessary to inject a small volume of air through the cannula and into the intrapleural space before pressure recording can be initiated.

The lightness and flexibility of the polyethylene cannula permit it to ride along easily with thoracic movements and once properly positioned within the pleura it continues to transmit pressures faithfully for long periods of time. At the conclusion of our experiments we routinely perform a thoracotomy to confirm location of the cannula. Gross examination of the lungs at this time reveals little or no damage to pulmonary tissue resulting from the cannulation procedure.

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REFERENCES

NADEL, J. A. & WIDDICOMBE, J. G. (1962). *J. Physiol., Lond.*, **163**, 13–33.
AMDUR, M. O. & MEAD, J. (1955). *Proc. 3rd Nat. Air Poll. Symp. Pasedena*, 150–159.

## Effect of temperature changes on the tone of perfused mesenteric arteries of rat and on the perfusion pressure responses to sympathetic nerve stimulation and injected noradrenaline

Exposure of cat cutaneous vessels to cold reduces the vasoconstrictor response to nerve stimulation and injected catecholamines (Folkow, Fox & others, 1963). Rogers, Atkinson & Long (1965), using the perfused mesenteric arteries of dog, found that a decrease in temperature of the perfusion fluid from 37° to 27° increased the basal-line perfusion pressure and the responses to nerve stimulation and injected catecholamines. They suggested that it might be due to the decreased luminar diameter at lower temperatures which may have resulted in increased physical efficiency of the vascular smooth muscle and this in turn increased the perfusion pressure responses to nerve stimulation and intra-arterially injected catecholamines.

The present study on the mesenteric arteries of rat perfused with Tyrode solution indicate that responses to sympathetic nerve stimulation and injected noradrenaline are affected differently with change of temperature of the perfusion fluid.

Female albino rats, weighing 250–300 g were used. The superior mesenteric artery was isolated, cannulated and removed together with its small resistance vessels as described by McGregor (1965). A Harvard peristaltic pump (Harvard Apparatus Co., Model 1210) was used to perfuse the cannulated artery with Tyrode solution at a constant flow of 25 ml/min. The solution was gassed with 5% carbon dioxide in oxygen. The temperature of the solution was changed by cooling or heating the water circulating around the perfusion coil. The responses were recorded manometrically using a frontal writing lever. Before cannulation of the artery, when the pump was operating and the flow was 25 ml/min, the pressure was 50 mm Hg. When the vessels were being perfused at the same rate the pressure was 85 mm Hg. Thus the average basal perfusion pressure during an experiment was 25 mm Hg.

The perivascular nerves were stimulated using a bipolar platinum electrode with a Grass stimulator (biphasic rectangular pulses of 20 V; 1 ms; at 7/s). Noradrenaline was injected directly into the cannula leading to superior mesenteric artery over a period of 5 s by Palmer pump (F-30).

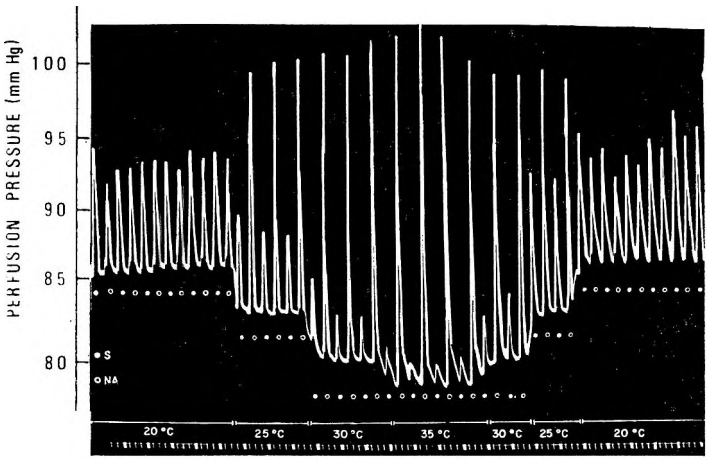


FIG. 1. Effect of temperature changes on the tone of perfused mesenteric arteries of rat and on the perfusion pressure responses to sympathetic nerve stimulation and injected noradrenaline. The mesenteric arteries were perfused with Tyrode solution at a constant flow of 25 ml/min at different temperatures. The perivascular sympathetic nerves (S) were stimulated using biphasic rectangular pulses (20 V; 1 ms; at 7/s) every 4 min for 20 s. Noradrenaline (NA 3 $\mu$g) was injected directly into the cannula leading to superior mesenteric artery by Palmer pump (F-30). The increase in temperature of the perfusion fluid from 20° to 35° decreased the tone. The response to nerve stimulation was reduced and that to injected NA enhanced. Lowering the temperature of the perfusion fluid from 35° to 20° increased the tone, and the response to nerve stimulation enhanced while that to injected NA reduced.

The perfusion pressure responses to both sympathetic nerve stimulation and injected noradrenaline in all experiments were submaximal. An amount of noradrenaline (3–5 $\mu$g) was injected which produced a vasoconstrictor response of equal magnitude to that produced by sympathetic nerve stimulation (20°). After recording a few control responses to nerve stimulation and injected amine at a fixed temperature of 20° or 35°, the temperature of the perfusion fluid was gradually changed and further responses recorded. Fig. 1 illustrates an example. The increase in temperature from 20° to 35° decreased the tone. The response to nerve stimulation was reduced while that to injected noradrenaline was enhanced. When the temperature was lowered again from 35° to 20° the tone was increased, and the response to nerve stimulation was potentiated while that to injected noradrenaline was reduced. From these experiments it is evident that the response of mesenteric arteries of rat to sympathetic nerve stimulation and injected noradrenaline are affected differently with the change of temperature of the perfusion fluid and thus cannot be explained simply by the change in tone produced by varying the temperature of the perfusion fluid.

I wish to thank Dr. G. M. Ling for providing the facilities to carry out this work. This work was supported by the Ontario Mental Health Foundation (OMHF).

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REFERENCES

FOLKOW, B., FOX, R. H., KROG, J., ODELRAM, H. & THORÉN, O. (1963). *Acta physiol. scand.*, **58**, 342–354.

MCGREGOR, D. D. (1965). *J. Physiol., Lond.*, **177**, 21–30.

ROGERS, L. A., ATKINSON, R. A. & LONG, J. P. (1965). *Am. J. Physiol.*, **209**, 376–382.

# Effect of aminophylline, butalamine and imolamine on human isolated smooth muscle

Butalamine, (3-phenyl-5-dibutylaminoethyl-amino-1,2,4-oxadiazole) and imolamine, (3-phenyl-5-imino-4-diethylaminoethyl-1,2,4-oxadiazole), have been shown to produce in animals coronary vasodilation, local anaesthesia, analgesia and a papaverine like action in duodenal preparations (Sterne & Hirsch, 1964, 1965). We have now investigated the spasmolytic activity of these drugs on isolated, spontaneously-active, human smooth muscle tissue and compared them with aminophylline. The tissue was fresh from operation specimens. Strips were prepared and suspended in an organ bath containing Krebs bicarbonate solution at 37° gassed with 5% carbon dioxide in oxygen. The effects of the drug on the rate of spontaneous activity, the amplitude of contractions and tone were recorded on a smoked kymograph drum. The results are in Table 1. The anti-acetylcholine properties of the compounds were also investigated by measuring the reduction in height of acetylcholine-induced contractions.

From Table 1, it can be seen that butalamine, like aminophylline, caused a slowing in rate of spontaneous activity, a decrease in amplitude and a reduction in tone of the tissues studied. Butalamine appeared more potent on ileum and one piece of uterus but it was otherwise approximately equipotent with aminophylline. Imolamine increased the tone of uterus and ileum and this was accompanied by a reduction in amplitude of contraction. The response of the stomach tissue to imolamine was similar to that of butalamine and aminophylline, i.e. a relaxant action on smooth muscle. Butalamine has a more potent anti-acetylcholine activity than imolamine. Butalamine (10 $\mu$g/ml final bath concn) reduced contractions of the uterus preparation by 90% while imolamine (1 and 10 $\mu$g/ml) gave no response. Both drugs at 4 $\mu$g/ml caused a 20% reduction in the contractions of the longitudinal muscle of the appendix. Butalamine (10 $\mu$g/ml) caused a 50% reduction and imolamine (10 $\mu$g/ml) a 20%

Table 1. *The effect of aminophylline, butalamine and imolamine on the spontaneous activity of isolated human smooth muscle*

| Tissue | No of expts | Drug | Dose $\mu$g/ml (final bath concn) | Effect on: Rate of spontaneous activity | Effect on: amplitude of contraction | Effect on: tone |
|---|---|---|---|---|---|---|
| Uterus Hysterectomy | 3 | Aminophylline | 2·5–10 | Slowed | No change | Decreased |
| | | Butalamine | 1 | No change | No change | No change |
| | | | 10 | Slowed | No change | Relaxation |
| | | Imolamine | 10 | No change | Increased | No change |
| | | | 50 | No change | Reduced | Increased |
| | | | 100 | Slowed | Reduced | No change |
| Uterus Hysterectomy | 1 | Aminophylline | 500–2 mg | No change | No change | Decreased |
| | | Butalamine | 25–200 | No change | No change | Decreased |
| | | Imolamine | 25–200 | No change | No change | Increased |
| Ileum Circular muscle | 2 | Aminophylline | 25–100 | Slowed | No change | Decreased |
| | | | 200 | Slowed | Reduced | Decreased |
| | | | 400–500 | Abolished | Abolished | Decreased |
| | | Butalamine | 100 | Abolished | Abolished | Decreased |
| | | Imolamine | 100 | No change | Reduced | Increased |
| Stomach Longitudinal muscle | 1 | Aminophylline | 25 | Slowed | No change | Decreased |
| | | | 100 | Abolished | Abolished | Decreased |
| | | Butalamine | 50–100 | Abolished | Abolished | Decreased |
| | | Imolamine | 100 | Abolished | Abolished | Decreased |

reduction of the contractions induced in the stomach preparation. This supports the results of one further separate experiment on longitudinal stomach strips where imolamine (10 $\mu$g/ml) did not alter the acetylcholine dose-response curve but butalamine (10 $\mu$g/ml) caused a shift to the right and a flattening of the curve.

From these preliminary experiments, it would appear that butalamine is a more effective smooth muscle relaxant compound than imolamine. It has a similar potency to aminophylline on isolated human smooth muscle. Imolamine has a variable action on tone, producing an increase in ileum and uterus and a decrease in stomach.

We thank our surgical colleagues for providing us with the operation specimens used in this study. The work has been supported by a grant from the Board of Governors of St. Bartholomew's Hospital. Butalamine and imolamine were supplied by Rona Laboratories Ltd.

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REFERENCES

STERNE, J. & HIRSCH, C. (1964). *IVth European Congress of Cardiology, Prague.*
STERNE, J. & HIRSCH, C. (1965). *Thérapie*, **20**, 89–94; 95–100.

# Protection against *m*-fluorotyrosine convulsions and lethality in mice exposed to hypobaric hypoxia

Acute exposure to hypoxia shifts brain metabolism to anaerobic pathways (Gurdjian, Webster & Stone, 1949) and elevates $\gamma$-aminobutyric acid (Wood, Watson & Ducker, 1968). Drugs which cause convulsions and impair aerobic metabolism or deplete brain $\gamma$-aminobutyric acid should therefore induce fewer convulsions during hypoxia.

Semicarbazide is thought to act in this way (Killam & Bain, 1957) and the convulsions it induced were antagonized by hypobaric hypoxia (Baumel, Shatz & others, 1969). *m*-Fluorotyrosine impairs oxidative metabolism in brain (Weissman & Koe, 1967), and we now show acute hypoxia to antagonize the convulsions and mortality it produces.

Swiss albino, random-bred male mice (Charles River Farms), 22–26 g were housed at 21–23° with room lights alternating on a 12-h light-dark cycle. The hypobaric chambers (Baumel, Robinson & Blatt, 1967) were plexiglass desiccators (internal diameter 10 in, height 14 in) connected, in parallel, to a vacuum pump through a manifold which exhausted room air.

Drug solutions were freshly prepared immediately before intraperitoneal injection. The animals were injected and immediately placed, in pairs, in the four altitude-chambers which were then decompressed over a 10-min period to 364 mm Hg (10% $O_2$). Controls were placed in identical chambers open to room air (760 mm Hg, 21% $O_2$).

Hypobaric hypoxia protected against *m*-fluorotyrosine convulsions at 3 and 4 h after administration of the drug. Lethality was decreased throughout the exposure period (Fig. 1).

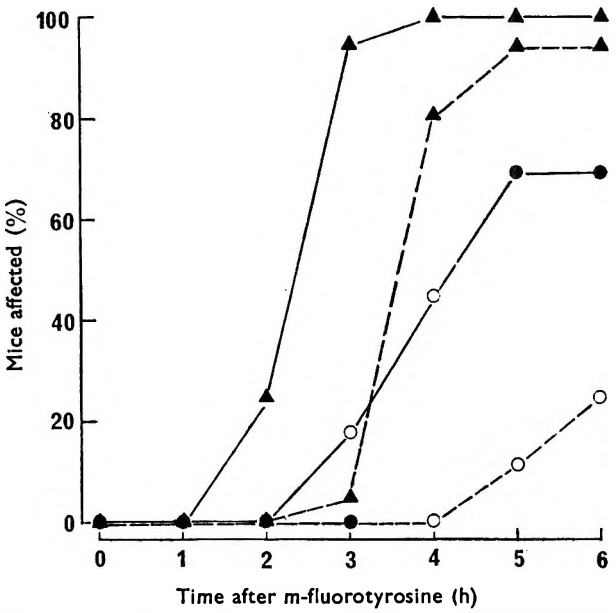


FIG. 1. Effect of hypobaric hypoxia on *m*-fluorotyrosine (10 mg/kg intraperitoneally) convulsions (—) and lethality (- - -). ▲ Sea level. ● Hypobaric. Clear circles denote significant difference ($P < 0{\cdot}005$) from sea level.

The similarity of the acute effects of *m*-fluorotyrosine to those of fluoroacetate in several species (Chenoweth, 1949; Pattison, 1959) and accumulation of citric acid in brain and kidney of mice (Weissman & Koe, 1967) suggests that metabolism to fluoroacetate and blockade of the Krebs cycle by this metabolic poison is the mechanism of convulsions produced by *m*-fluorotyrosine. Hypoxia may antagonize *m*-fluorotyrosine convulsions by one or more of three probable mechanisms: hypoxia may decrease the rate of conversion of *m*-fluorotyrosine to fluoroacetate; increased dependence of neurons on anaerobic metabolism during hypoxia may reduce the consequences of impairing aerobic metabolism by fluoroacetate, or a rise in brain $\gamma$-aminobutryic acid during hypoxia (Wood, Watson & Ducker, 1968) may increase the threshold of vulnerable neurons to seizure activity.

This investigation was supported by PHA Training Grant No. 1T01ES 00104 from the Division of Environmental Health Sciences.

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REFERENCES

BAUMEL, I., SCHATZ, R., DEFEO, J. J. & LAL, H. (1969). *J. Pharm. Pharmac.*, **21**, 119–120.
BAUMEL, I., ROBINSON, S. M. & BLATT, W. F. (1967). *J. pharm. Sci.*, **56**, 918–919.
CHENOWETH, M. B. (1949). *Pharmac. Rev.*, **1**, 383–424.
GURDJIAN, E. S., WEBSTER, J. E. & STONE, W. E. (1949). *Am. J. Physiol.*, **156**, 149–157.
KILLAM, K. F. & BAIN, J. A. (1957). *J. Pharmac. exp. Ther.*, **119**, 255–252.
PATTISON, F. L. M. (1959). *Toxic Aliphatic Fluorine Compounds.* Amsterdam: Elsevier.
WEISSMAN, A. & KOE, K. B. (1967). *J. Pharmac. exp. Ther.*, **155**, 135–144.
WOOD, J. D., WATSON, W. J. & DUCKER, A. J. (1968). *J. Neurochem.*, **15**, 603–608.

# Gas chromatographic evaluation of 5,5-dimethyl-2,4-oxazolidinedione (DMO) for determination of intracellular pH

The DMO technique is an effective way of calculating the total body and skeletal muscle intracellular pH (Longhini, Gallitelli & others, 1968; Schloerb & Grantham, 1965; Irvine & Dow, 1966; Constant, 1967). DMO evaluation is usually made using plasma, muscle and urine by the Waddell and Butler ultraviolet spectrophotometric method (Butler, 1953; Waddell & Butler, 1957, 1959), unless [2-$^{14}$C]5,5-dimethyl-2,4-oxazolidinedione is used (Schloerb & Grantham, 1965; Irvine & Dow, 1966).

In the gas-liquid chromatographic method DMO is first extracted, according to the Waddell & Butler technique (Butler, 1953; Waddell & Butler, 1957, 1959), the final solution from this method, i.e. in 0·05M borate buffer, pH 9, being acidified by 5N HCl to pH 1–1·5 and then extracted by peroxide-free ethyl ether four times. The ethyl ether extracts are dried over $Na_2SO_4$ and evaporated at 35° in a flow of nitrogen and the residue chromatographed (10% butandiol succinate on Gas-chrom Q 90–100 mesh, 2 m × 2 mm i.d. stainless steel, column temperature 213°, 20 ml $N_2$/min, DMO retention time relative to that of the internal standard, 5-methyl-2,4-oxazolidinedione, 0·64; detector: flame ionization 130°; injector: 265°. Both compounds are synthesized according to Stoughton (1941).

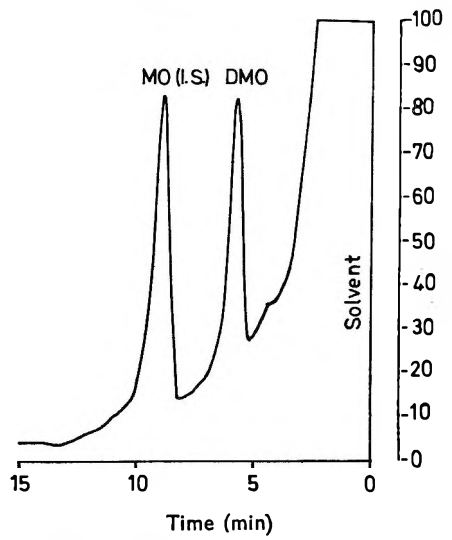


FIG. 1. Gas-chromatogram of DMO from human muscle sample. Analytical details are given in the text.

The tests on DMO value in blank, plasma, urine and skeletal muscle homogenates are in agreement with those obtained by us using the Waddell & Butler method. The chromatographic method, however, is sensitive (about 1 $\mu$g) and specific (Fig. 1) enough to enable DMO evaluation on human muscle samples obtained by needle-biopsy. This avoids the use of the expensive [2-$^{14}$C]5,5-dimethyl-2,4-oxazolidinedione.

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REFERENCES

BUTLER, T. C. (1953). *J. Pharmac. exp. Ther.*, **108**, 11–17.
CONSTANT, M. A. (1967). *Invest. Ophtal.*, **6**, 484–497.
IRVINE, R. O. H. & DOW, J. (1966). *Clin. Sci.*, **31**, 317–324.
LONGHINI, E., GALLITELLI, L., SANTABROGIO, S. & SARDINI, D. (1968). *Min. Med.*, **59**, 4418–4433.
SCHLOERB, P. R. & GRANTHAM, J. J. (1965). *J. Lab. clin. Med.*, **65**, 669–676.
STOUGHTON, R. W. (1941). *J. Am. chem. Soc.*, **63**, 2376–2379.
WADDELL, W. J. & BUTLER, T. C. (1957). *Proc. Soc. exp. Biol. Med.*, **96**, 563–565.
WADDELL, W. J. & BUTLER, T. C. (1959). *J. clin. Invest.*, **38**, 720–729.

# Is there a simple explanation for the sensitization to histamine produced by adrenergic β-receptor antagonists?

The high resistance of mice to the lethal effects of histamine can be reduced by *Bacillus pertussis* vaccine (Parfentjev & Goodline, 1948; Munoz & Bergman, 1966) or by adrenergic β-receptor antagonists (Fishel, Szentivanyl & Talamage, 1962; Townley, Trapani & Szentivanyl, 1967; Bergman & Munoz, 1968). On this basis, Fishel, Szentivanyl & Talamage (1964) have argued that the lethal effects of histamine in mice are usually antagonized by the simultaneous release of large amounts of catecholamines; when *B. pertussis* vaccine or other drugs block the adrenergic β-receptors, lethality is believed to have been increased by unbalanced α-receptor stimulation. This explanation is vulnerable because it has also been shown that histamine lethality is enhanced by adrenalectomy (Halpern & Wood, 1950) when a reduction in both α- and β-receptor stimulation would be expected, and that the enhanced lethality produced by a β-receptor antagonist can be reversed by a large dose of adrenaline (Bergman & Munoz, 1966, 1968) when even greater α-receptor stimulation would be expected. There is, of course, a simpler explanation which would resolve these difficulties. It is conceivable that histamine produces death in mice by acute bronchoconstriction and that this action is usually attenuated by the bronchodilator action of histamine-released catecholamines. Before attempting to evaluate this hypothesis directly, it seemed important to assess the potential effects of the non-specific actions of adrenergic β-receptor antagonists. To do this, the effects of both (+)- and (−)-isomers of D-(−)-2-isopropylamino-1-(*p*-nitrophenyl)ethanol (INPEA) (Almirante & Murmann, 1966) have been studied.

Differences in sensitivity to histamine due to environmental conditions and strain differences are known to exist. For this investigation, therefore, 2 random-bred strains (NMRI and AP-1) and 5 inbred strains (C3H/He/Sel, CE/Sel, DBA/2/Sel, C57L/Sel, C57Bl/10/Sel) were used with the aim of finding the most suitable strain. All the animals, including the inbred strains, come from our breeding station where they were kept solely on a diet of Rieper/MT pellets and deionized water. The environmental conditions in the laboratory were the same as in the animal quarters (25° ± 0·3). The animals had been fasted for 18 h before testing. To measure the sensitivity to histamine, groups of 10 adult male mice of each strain were given, intraperitoneally, doses of histamine HCl in saline corresponding to 15, 60 and 600 mg/kg histamine base. D-(−)-INPEA, L-(+)-INPEA and propranolol were injected intravenously at various doses in 0·2 ml saline at the rate of 0·01 ml/s, 15 min before the histamine challenge. Mice sensitive to histamine showed sedation, cyanosis, defeacation, unsteady gait and respiratory distress. Many of these animals convulsed and died within 5–20 min of the challenge injection. Only the 24 h toxicity value of histamine was estimated.

All the strains used showed the usual high resistance to the lethal effects of histamine and different animals in any strain varied greatly, both in the effects produced by

histamine and their sensitivity to the drug. Thus, no clear dose-activity relation could be established in any strain. In fact, the percentage of control animals that died after a challenge with 60 mg/kg was not significantly different from that observed after a challenge with 600 mg/kg. In 6 out of 7 strains, the control lethality observed was always between 0–10%.

In an attempt to investigate the dose-activity relations of the agents under examination in a first series of experiments, groups of NMRI mice were challenged with standard doses of histamine, corresponding to 15, 60 and 600 mg/kg histamine base, respectively, 15 min after treatment with graded doses of the agents under examination or saline. The results obtained are reported in Table 1.

Table 1. *Effects of* D-(−)- *and* L-(+)-INPEA *on histamine toxicity in NMRI mice*

| Dose | Histamine (base) challenge 15 mg/kg i.p. | | | Histamine (base) challenge 60 mg/kg i.p. | | | Histamine (base) challenge 600 mg/kg i.p. | | |
|---|---|---|---|---|---|---|---|---|---|
| mg/kg i.v. | D-(−)-INPEA | L-(+)-INPEA | Propranolol | D-(−)-INPEA | L-(+)-INPEA | Propranolol | D-(−)-INPEA | L-(+)-INPEA | Propranolol |
| 1 | 0/20* 0 | 2/20 10 | 4/30 13 | 10/20 50 | 1/20 5 | 9/20 45 | 6/20 30 | — | 3/30 10 |
| 6 | 2/20 10 | 2/20 10 | 8/20 40 | 8/20 40 | 1/20 5 | 9/20 45 | 12/30 40 | 14/30 47 | 18/30 60 |
| 12 | 3/20 15 | 2/20 10 | 12/20 60 | 13/20 65 | 2/20 10 | 16/20 80 | 16/40 40 | 8/40 20 | 16/30 53 |
| 24 | 7/20 35 | 2/20 10 | — | 14/20 70 | 2/20 10 | — | 19/40 48 | 21/40 53 | 24/30 80 |
| 48 | — | — | — | — | — | — | 25/30 83 | 27/30 90 | — |
| Saline | | 1/50 5 | | | 4/50 8 | | | 4/40 10 | |

* Death/number and percentage of death of mice tested.

Table 2. *Effects of* D-(−)- *and* L-(+)-INPEA *on histamine toxicity in* 6 *different strains of mice*

| Histamine (base) challenge mg/kg i.p. | Agent | Dose mg/kg i.v. | AP-1* | C3H/He/Sel | CE/Sel | DBA/2/Sel | C57L/Sel | C57B1/10/Sel |
|---|---|---|---|---|---|---|---|---|
| 60 | D-(−)-INPEA | 12 | 4/10† 40 | 3/10 30 | 0/10 0 | 0/20 0 | 4/7 57 | 2/10 20 |
| | L-(+)-INPEA | 12 | 5/10 50 | 1/10 10 | 0/10 0 | 0/20 0 | 1/7 14 | 0/10 0 |
| | Propranolol | 6 | 9/10 90 | 4/10 40 | 3/10 30 | 3/20 15 | 7/7 100 | 4/10 40 |
| | Saline | — | 0/10 0 | 0/10 0 | 0/10 0 | 0/20 0 | 0/7 0 | 0/10 0 |
| 600 | D-(−)-INPEA | 12 | 8/10 80 | 4/10 40 | 7/10 70 | 7/10 70 | 7/7 100 | 3/10 30 |
| | L-(+)-INPEA | 12 | 8/10 80 | 0/10 0 | 8/10 80 | 6/10 60 | 5/7 72 | 1/10 10 |
| | Propranolol | 6 | 7/10 70 | 3/10 30 | 6/10 60 | 4/10 40 | 7/7 100 | 4/10 40 |
| | Saline | — | 1/10 10 | 1/10 10 | 0/10 0 | 1/10 10 | 2/7 29 | 0/10 0 |

* Strain used.
† Death/number and percentage of death of mice tested.

In the NMRI mice challenged with 15 and 60 mg/kg of histamine, both propranolol, and to a somewhat lesser extent D-(−)-INPEA, enhanced the lethal effects of histamine, while L-(+)-INPEA was without effect. After challenge with 600 mg/kg of histamine, on the other hand, all three agents markedly potentiated histamine toxicity. It is apparent that the mechanism of the sensitizing action observed after challenge with 15 and 60 mg/kg histamine differs from that seen after challenge with 600 mg/kg. These observations are compatible with the assumption that the effect seen after the lower challenges is due to $\beta$-adrenergic receptor blockade; the effect seen in the highly challenged mice, on the other hand, does not appear to have such a simple explanation.

In a second series of experiments, groups of male mice of 6 different strains were challenged with two doses of histamine, i.e. 60 and 600 mg/kg (histamine base), respectively, 15 min after injecting a standard dose of the test agents. The results obtained are in Table 2.

Some interesting patterns emerged from this investigation. As was found with NMRI mice, histamine alone was not more toxic to mice of any strain (except perhaps strain C57L/Sel) after 600 mg/kg than after 60 mg/kg intraperitoneally. However, evidence of strain differences to histamine sensitization was found, especially in the mice challenged with 80 mg/kg of histamine. Strain C57L/Sel seemed to be most sensitive to histamine potentiation by $\beta$-adrenergic blockade, but the other

inbred strains too, showed some histamine sensitization after propranolol. The behaviour of D-(−)-INPEA in these experiments perhaps could be explained by the lower adrenergic β-blocking potency of this agent compared with that of propranolol. L-(+)-INPEA, again, was ineffective. Random-bred strain AP-1, however, after challenge with 80 mg/kg of histamine, behaved in exactly the same manner as observed after challenge with 600 mg/kg, indicating a particular sensitivity to histamine sensitization. In all other strains, and unlike the response to 60 mg/kg of histamine, all three agents produced about the same toxic effect after 600 mg/kg histamine. No significant difference could be seen between the enhancing effect of the β-blockers D-(−)-INPEA and propranolol and that of the non-blocking isomer L-(+)-INPEA. There is thus a suggestion of a completely different mechanism of action.

Some conclusions concerning the mechanism of action can be drawn. In the mice challenged with the low doses of histamine, the behaviour of D-(−)-INPEA runs parallel with that of propranolol. Consequently, the same interpretation could apply; that there is a good correlation between β-receptor blockade and sensitization to histamine toxicity. Nevertheless, it is puzzling that although D-(−)-INPEA and propranolol were given at doses causing highly effective blockade of β-adrenergic receptors, in only one strain (C57L/Sel) was a 100% mortality to the challenge of histamine observed. This is at variance with the results of Bergman & Munoz (1968) who still observed 100% lethality in their CFW mice with doses of propranolol much lower than those we used, and this could mean that the same individual differences in the sensitivity to histamine might also exist for the histamine-sensitizing effect of other drugs. This would indicate that even complete β-blockade is not capable of sufficiently sensitizing those animals that are particularly resistant to histamine. Strain C57L/Sel on the other hand, might be particularly sensitive to histamine and consequently give results more like those described by Bergman & Munoz (1968) with CFW mice.

The mechanism by which sensitivity of pretreated mice to very high doses of histamine was raised is not clear. Since D-(−)- as well as L-(+)-INPEA were equally effective, the sensitization to histamine toxicity cannot be explained by actions on the adrenergic β-receptors. These data indicate clearly that at high histamine challenge the interference would be unspecific. Again, it is interesting that even with massive doses of histamine only occasionally was 100% lethality observed.

In conclusion, it seems unlikely that a simple explanation can be given at present to explain the effects of drugs in increasing the lethality to histamine.

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March 3, 1969

## REFERENCES

ALMIRANTE, L. & MURMANN, W. (1966). *J. mednl Chem.*, **9**, 650–653.

BERGMAN, R. K. & MUNOZ, J. (1966). *Proc. Soc. exp. Biol. Med.*, **122**, 428–433.

BERGMAN, R. K. & MUNOZ, J. (1968). *Nature, Lond.*, **217**, 1173–1174.

FISHEL, C. W., SZENTIVANYL, A. & TALAMAGE, D. W. (1962). *J. Immunol.*, **89**, 8–18.

FISHEL, C. W., SZENTIVANYL, A. & TALAMAGE, D. W. (1964). In *Bacterial Endotoxins*, Editors: Landy, M. & Braun, W., p. 474. Rahway, New Jersey: Quinn & Boden Co.

HALPERN, B. N. & WOOD, D. (1950). *C.r. hebd. Séanc. Acad. Sci. (Paris)*, **230**, 138–140.

MUNOZ, J. & BERGMAN, R. K. (1966). *J. Immunol.*, **97**, 120–125.

PARFENTJEV, I. A., & GOODLINE, M. A. (1948). *J. Pharmac. exp. Ther.*, **92**, 411–413.

TOWNLEY, R. G., TRAPANI, I. L. & SZENTIVANYL, A. (1967). *J. Allergy*, **39**, 177–197.

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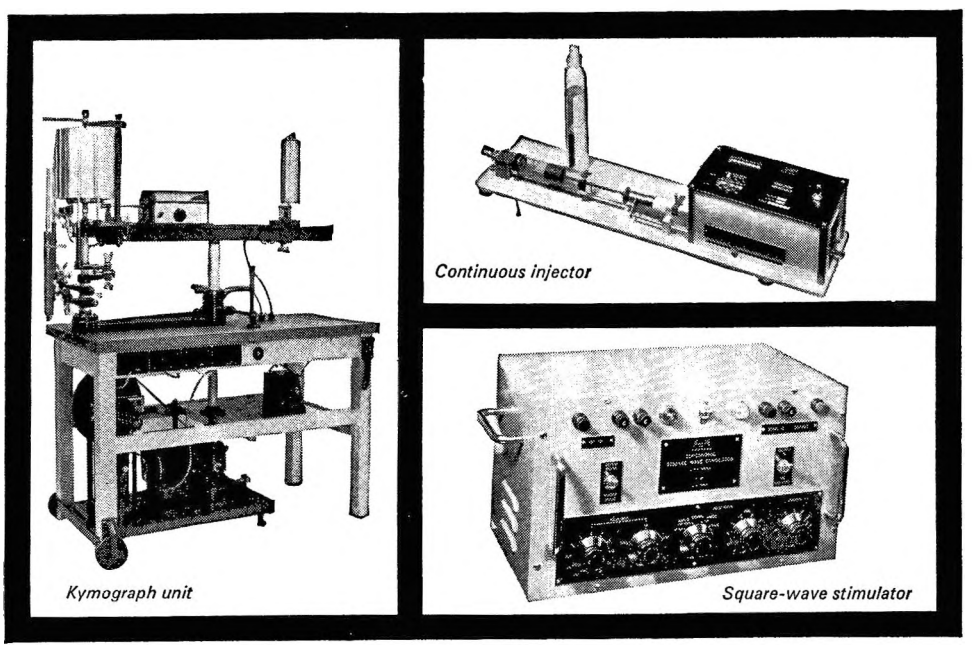

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**Printed by W Heffer & Sons Ltd Cambridge England**

Journal of Pharmacy and Pharmacology

Volume 21 Number 7 July 1969

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